

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES

VOLUME 64, ART. 5 PAGES 735-1073

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SECOND TISSUE HOMOTRANSPLANTATION CONFERENCE

BY

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NEW YORK

PUBLISHED BY THE ACADEMY

March 22, 1957

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(Founded in 1817)

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INTRODUCTORY REMARKS

By Hilary Koprowski

Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

The unfailing energy and persistent courage of John Marquis Converse and Blair O. Rogers made it possible to arrange the conference on which this monograph is based two years after the first meeting on the same general subject of tissue transplantation took place. In this monograph, in contrast to the first publication, greater emphasis is placed on basic experimental data and less on clinical material. Thus, though the title mentions only homotransplantation, the scope of the papers and discussions is much broader.

If this series of yearly meetings continues, and if the general rate of progress in this particular field is maintained, the future feats of plastic surgery may exceed those envisioned by the wildest imagination. Even the exploits of Baron Munchausen in the same sphere of activity will be surpassed. I refer especially to his description of an adventure¹ in which he tells how, after pursuing his enemy into a walled town, he walked his panting horse "to a spring in the market-place, and let him drink." I quote further, "He drunk uncommonly—with an eagerness not to be satisfied, but natural enough, for when I looked round for my men, what should I see, gentlemen? the hind part of the poor creature, croup and legs were missing, as if he had been cut in two, and the water run out as it came in, without either refreshing him or doing him any good. How it could have happened was quite a mystery to me, till I returned with him to the town gate. There I saw that when I rushed in peace meal with the flying enemy, they had dropt the portcullis, and unperceived by me, and the spirited animal, it had totally cut off his hind part, which lay still quivering on the outside of the gate. It would have been an irreparable loss, had not our farrier contrived to bring both parts together while hot. He sewed them up [with the help of plastic surgery, I presume] with sprigs and young shoots of laurels that were just at hand—the wound healed and what could not have happened, but to so glorious a horse, the [transplanted] sprigs took root in his body, grew up, and formed a bower over me, so that afterwards I could go upon many other expeditions in the shade of my own and my horse's laurels."

The only thing the indomitable Baron failed to do was to coin a scientific term for this type of transplantation. Was the laurel graft in the horse's hide an isotransplant, a homotransplant, or a heterotransplant?

Reference

1. RASPE, R. E. and others [*sic*]. 1948. Singular Travels, Campaigns and Adventures of Baron Munchausen. J. Carswell, Ed. Cresset Press. London, England.

INTRODUCTION

*By John Marquis Converse and Blair O. Rogers
New York University College of Medicine, New York, N. Y.*

The brief history of international conferences organized to report and discuss the results achieved by research workers in the field of tissue homotransplantation begins with a conference held at Arden House, Harriman, N. Y., in October 1952. The general discussion at this first meeting was followed by the Ciba Foundation Conference on the Preservation and Transplantation of Normal Tissues in London, England, in March 1953.

In February 1954, the First Tissue Homotransplantation Conference was held under the auspices of The New York Academy of Sciences. The theme of this first conference was "The Relation of Immunology to Tissue Homotransplantation."

The Second Tissue Homotransplantation Conference, held in February 1956, was also held under the sponsorship of The New York Academy of Sciences. The emphasis at this meeting was placed on the use of embryonic, fetal, and neonatal donor tissues. The reports of workers in this field dealt with such subjects as the abrogating, paralyzing, enhancing, or altering of normal host-graft relationships.

The general progress made in the field of tissue homotransplantation and the enthusiasm for a continuation of these conferences leads one to anticipate the reporting of still more progressive data in the next or Third Conference being planned for 1958.

Part I. Genetics, Phylogenetics, and Acquired Tolerance

THE GENETICS OF SKIN GRAFTING*

By E. J. Eichwald, C. R. Silmsen, and N. Wheeler

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Knowledge of the genetics of tissue transplantation is based almost wholly on studies with tumors. These studies have resulted in the formulation of certain principles often referred to as Snell's laws.⁴ These laws state in part that (1) transplants within a single inbred strain ($A \rightarrow A$) grow progressively in all hosts; (2) transplants between strains ($A \rightarrow B$) do not grow, or grow temporarily and then regress; (3) transplants into F_1 hybrid animals where one parent was inbred and susceptible ($A \rightarrow AB$) grow progressively in all hosts; and (4) transplants originating in an F_1 hybrid will grow in all F_1 hybrids ($AB \rightarrow AB$) but *not* in members of either parent strain ($AB \rightarrow A$, $AB \rightarrow B$). These laws resemble the general principles of blood group compatibility: type A blood is compatible with type A ($A \rightarrow A$), but not with type B recipients; it is also compatible with type AB ($A \rightarrow AB$) recipients; type AB blood, however, is not compatible with type A or type B recipients ($AB \rightarrow A$, $AB \rightarrow B$).

The common underlying principle is that cells can be successfully transferred to a recipient having at least all (genetically controlled, dominant) isoantigens present in the transferred cells. Additional isoantigens in the recipient matter little, but the lack of a donor isoantigen in a recipient will give rise to an immune response resulting in rejection or destruction of the donated cells.

It has often been assumed that these laws apply equally to the transplantation of other normal cells.^{1,2} This assumption has not been put to a rigid test. In the course of a study of the histocompatibility genes influencing the antigenic constitution, or transplantability, of mouse skin, we encountered many instances of graft failure that should not have occurred if Snell's laws, as formulated above, had applied fully. Analysis of these failures has led us to suspect the presence of a heretofore unsuspected histocompatibility gene on the Y chromosome of male mice. The effects of this gene, while readily consistent with the basic principle of Snell's laws, are in contradiction to some of its postulates referred to above.

Material and Methods

Mice of the A/Jax and C57BL strains and their F_1 hybrids, originally obtained from the R. B. Jackson Laboratories, Bar Harbor, Maine, and maintained by rigid brother-sister matings, served as donors and recipients of skin. The technique of transplantation has been previously described.³ Full-thickness tail skin was placed on the body wall in a suprapannicular bed accommodating an autograft and a test graft. Successful grafts were followed for a period in excess of 4 months. The mean survival time of interstrain grafts was between 11 and 12 days.

* These studies were conducted with the support of a grant (C 2455) from the United States Public Health Service, Bethesda, Md., a grant from the Montana Division of the American Cancer Society, New York, N. Y., and support from the Montana Deaconess Hospital Research Fund, Great Falls, Mont.

TABLE 1
INCIDENCE OF SUCCESSFUL SKIN GRAFTS, INTRA-STRAIN AND TO F₁ HYBRIDS
(Success/total)

	♂ to ♂	♀ to ♀	♀ to ♂	♂ to ♀
A/Jax to A/Jax.....	10/10	19/19	4/4	10/24
C57BL to C57BL.....	12/12	17/18	7/8	0/22
A/Jax to F ₁	17/18	7/7	13/14	0/18
C57BL to F ₁	7/7	7/7	13/13	0/13
F ₁ to F ₁	15/15	12/12	9/9	0/4
Totals.....	61/62	62/63	46/48	10/81

Observations

The data in TABLE 1 show that intrastrain grafts, grafts from pure strains to F₁ hybrids, and from one F₁ hybrid to another F₁ hybrid almost always succeeded, provided donor and recipient were of the same sex, or provided the donor was female and the recipient male. When the donor was male and the recipient female, grafts failed (FIGURE 1) except when both donor and recipient were of the A/Jax strain—in which case successes and failures were almost equal in number.

Failure of male grafts in female recipients suggests the existence of a histocompatibility gene on the Y chromosome of male mice. Since females (XX) lack the Y chromosome, while males (XY) carry both the X and the Y chromosome, a Y-linked gene would cause graft failure for the same reason that F₁ hybrid tumor cells are rejected by pure-strain parents and type AB erythrocytes are agglutinated by type A or type B recipients.

An alternate explanation for the failure of male grafts on females is the assumption that male grafts require an adequate level of male sex hormone, a requirement female animals do not meet. Female grafts, in contrast, would not require a corresponding level of female sex hormone.

Both assumptions, that of a Y-linked histocompatibility gene and of a hormonal requirement are readily tested. Histocompatibility genes, acting as isoantigens, immunize a recipient, as evidenced by a more rapid rejection of a second skin graft from the same or a genetically identical donor (second-set phenomenon). The mean rejection time of first-set male grafts by females of the same strain (or their F₁ hybrids) was 31 (±8) days. A second male skin graft from genetically identical donors was rejected in 13 (±0) days—evidence favoring the assumption of a Y-linked gene.

In an attempt to rule out the existence of a hormonal mechanism, male grafts were placed on 3 groups of female mice, one of which had been castrated, another placed on testosterone, and another had received testicular grafts from male mice of the same strain. A group of male mice received male skin grafts after having been castrated and placed on estrogen. The results were not clear-cut. Several of the female mice rejected, while the males accepted, the male skin as if no pretreatment had taken place. In several of the testosterone-treated females the grafts survived longer than expected. In one of the cas-

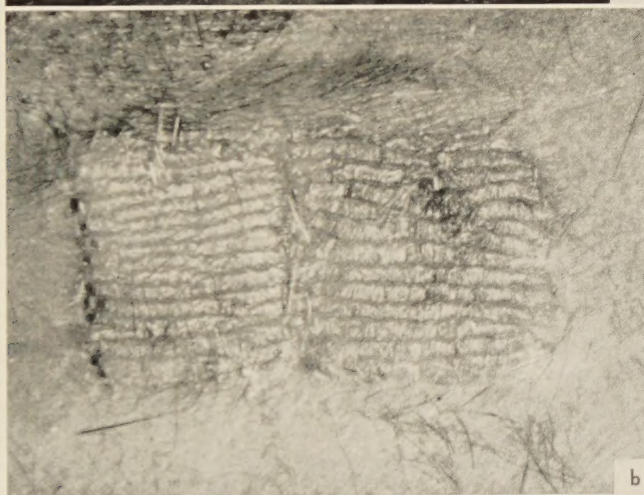
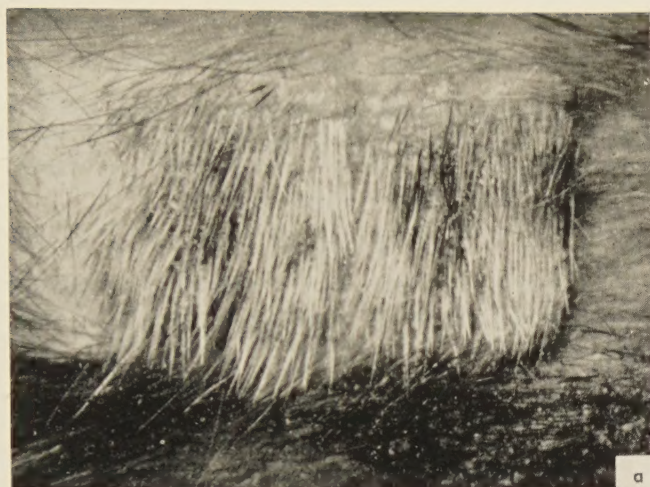


FIGURE 1. (a) Isograft from a male C57BL mouse on female C57BL mouse, one month after grafting; isograft at right, control autograft at left. (b) Same, clipped; note small erosion in center of isograft, an early indication of failure. (c) Same, 45 days after grafting, isograft having been replaced by a small pitted scar.

trated females the male graft persists as this report is being made—a period in excess of 4 months. Graft survival was shortened in all females having received testicular isografts. This probably represents a second-set phenomenon, the testicular tissue having served as the first-set.

Discussion

Failure of grafts within an inbred strain, and from an inbred animal to its F_1 hybrids, is not contrary to Snell's laws if these "laws" are considered mere applications of the one basic law that appears applicable to the transfer of tumors, erythrocytes, skin, and probably other tissues: that cells can be transferred successfully to recipients carrying at least the same effective histocompatibility genes as the transferred cells.

The presence of a histocompatibility gene in a graft, and its absence in the recipient, does not necessarily herald rejection of the graft. This appears evident from the frequent persistence of male skin on female recipients in the A/Jax strain. The consistent failure of these grafts on female F_1 hybrids suggests that males of the A/Jax strain also carry a Y-linked gene, which expresses itself consistently only when the recipient female is an F_1 hybrid. Very likely this denotes a relative weakness of this gene in the A/Jax strain.

The hormonal experiments, conducted thus far only on a pilot basis, are difficult to interpret. The possibility should be kept in mind that hormonal mechanisms may modify a course of events largely determined by the distribution and strength of histocompatibility genes.

References

1. KALISS, N. & I. ROBERTSON. 1943. Spleen transplantation relationships among two inbred lines of mice and their F_1 hybrid. *Genetics*. **28**: 78.
2. RANDALL, P. 1954. Communication. *Transplantation Bull.* **1**: 97.
3. SELMSER, C., C. R. BOND, & E. J. EICHWALD. 1955. The technique of mouse skin grafting. *Trans. Bull.* **2**: 38.
4. SNELL, G. D. 1953. Transplantable tumors. *In* *The Physiopathology of Cancer*. : 347. F. Homburger, Ed. Hoeber and Harper. New York, N. Y.

THE GENETICS OF SKIN HOMOTRANSPLANTATION IN THE HUMAN*

By Blair O. Rogers

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New York, N. Y.*

The majority of research on skin homografting and the biologic laws that may govern the behavior of skin homografts has been conducted in the past decade on animals. These experiments have often been performed in excellent laboratories, strict attention being paid to the use of adequate "control" material. Although there is a fairly substantial literature now available on the behavior of skin homografts in the human,¹⁻⁴ most of the clinical reports are concerned with the expedient use of skin homografts as temporary burn dressings. "Control" material in human experiments with skin homografting is relatively unknown in the medical literature to date. This report, however, is an initial attempt to rectify the scarcity of controlled human experiments in skin homografting. At the same time, it will deal with some of the genetic problems that arise whenever skin homografting is resorted to, and the possible significance that these problems may have on the field of tissue homotransplantation at large.

For many years surgeons have preferred to use members of the same family as donors of skin whenever homografting of a severely burned patient is required. It has seemed to be merely a matter of common sense and deduction that skin grafts taken from a closely related individual would be tolerated better by the burned host than those supplied by nonrelated individuals chosen at random from the general population. Moreover, anxious relatives of the burned patient are more easily persuaded to donate some of their own skin as graft material than casual friends or business associates of the burned victim.

When skin homografts supplied by nonrelated donors have been used, however, they too serve fairly well as a temporary burn dressing before they undergo the typical homograft rejection and slough. From the standpoint of survival time of genetically similar skin homografts in the human compared to the survival time of genetically dissimilar homografts, there has not been too much discussion in the literature to date. The merits of familial donors versus non-related donors, however, assume somewhat greater importance with the recent findings obtained from cross-skin homotransplantation in nonidentical twins.⁵⁻⁶ These findings seem to shed a new light on the greater advantage of using skin from members of the same or closely related families as donor material.

As a background to this problem, it is perhaps pertinent to give a brief résumé here of the recent important discoveries in skin homografting in the human. In 1927, K. H. Bauer⁷ of Heidelberg, Germany, reviewed the theoretical concepts of homografting that existed during the 1920's. Agreeing with

* The work partially described in this report is being supported in part by Research Grant No. RG-4788 from the National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

his predecessors Borst and Enderlen,⁸ Lexer,⁹⁻¹⁰ Perthes,¹¹ and Eden,¹² that the lack of permanent survival of homografts was caused by a hereditary or genetic difference between donor and host, Bauer postulated that homografting therefore would be successful, and similar to autografting, if donor and host were "genetically similar."

Only in identical twins could a "genetic similarity or equality" satisfy the requirements for his hypothesis. Nonidentical twins are presumably no more closely related than are ordinary full brothers and sisters.¹³ Bauer had the opportunity to operate upon a pair of identical twins whose genetic similarity was established by examination of blood groups and a close resemblance of physical characteristics. Both twins were born with the same form of syndactyly involving the fourth and fifth fingers of both hands. Bauer transplanted a piece of skin 1.2 by 5 cm. in size from one twin to the defect created on the fourth or ring finger during the syndactyly repair of the other twin. This homograft healed by primary intention and survived permanently, differing in no way from an ordinary autograft. Bauer had thus established for the first time that skin homografting could serve as a test for identical twinning. In subsequent years, a number of clinicians¹⁴⁻¹⁷ repeated Bauer's observations with identical results. McIndoe and Franceschetti¹⁸ summarized these reports and at the same time employed skin homografts in a remarkably simple yet ingenious manner to identify identical twins out of 3 children who had apparently been mistakenly exchanged soon after birth in the hospital nursery where all 3 children had been born. They describe this medicolegal problem in the following interesting excerpt: "In 1947 the parents of 6-year-old twins (Victor and Pierre J.) became aware of the existence of another small boy (Eric V.) who presented a striking resemblance to one of their own children. Believing first that it was simple coincidence, they were surprised to learn that the other child was born the same night, and in the same clinic as their own. During a parade in which the similarly dressed children were participating, the father was shocked by the resemblance and decided to contact the authorities in order to learn whether or not a substitution of one of his twins could have taken place." Full-thickness skin homografts transplanted between the two boys who had been raised together as twin brothers necrosed and sloughed off, whereas skin homografts between Victor and Eric, the true twins, survived permanently. "This story, unique both from the scientific and emotional point of view, had an interesting epilogue: in accordance with the conclusions of the investigation, the authorities ordered the exchange of the substituted children The adaptation of the children to their new environment is, as personal inquiries have confirmed, satisfactory."¹⁸

It is interesting to note that until recently⁵⁻⁶ there have been no reports in the medical literature on the behavior of skin homografts in nonidentical twins. Employing Bauer's reasoning, and the statement by Billingham *et al.*¹³ that nonidentical or 2-egg twins are genetically related to each other only as ordinary brothers and sisters, it occurred to us that skin homografts reciprocally transplanted between nonidentical twins, therefore, would eventually be rejected and sloughed as they invariably are when transplanted between ordi-

nary brothers and sisters. It should be mentioned here, in passing that, contrary to other clinicians,^{7, 14-19} Meyer-Burgdorff²⁰ described the rejection of skin homografts in a pair of twins whom he regarded as "identical" (monozygotic), but the exact methods of diagnosing this monozygosity were not listed in his brief text of 5 printed lines, and it seems more probable that these twins were actually nonidentical (dizygotic), or that his surgical technique was at fault.

Recently the opportunity arose reciprocally to transplant skin homografts in 4 sets of twins whose genetic identity had not been conclusively established. The results obtained were taken as evidence that nonidentical or dizygotic twins reject skin homografts reciprocally transplanted between them, thus contrasting with identical twins who accept each other's skin homografts permanently without any signs of rejection and sloughing.

Before describing these cases in detail, it would seem advisable at this point to outline briefly the routine methods used by the author and his colleagues in transplanting full-thickness skin homografts to human volunteers.

Methods

By employing a cork stopper or a metallic ring whose circumferential edge is painted with methylene blue, it is possible to imprint a uniform circle on the skin that serves as an outline of the graft to be excised. Full-thickness, circular skin homografts of identical size are then taken from and transplanted to the anterior thigh, volar forearm, or medial arm surfaces of human volunteers, depending upon their aesthetic or cosmetic preferences*. All visible subcutaneous fat is gently and carefully trimmed away from the dermal under-surface of the graft by means of small, straight plastic scissors.

Since the graft and the skin defect created by its removal are identical in size, all homografts transplanted by this method are "fitted" grafts. Many interrupted, 5-0 black silk bolus sutures are used to sew the graft into position. Each homograft is then dressed with vaseline, zeroform, or fine-mesh nylon gauze; the bolus is constructed of lamb's wool soaked in mineral oil or of absorbent cotton soaked in normal saline. Bulky, fluffed, dry, sterile gauze and elastic bandages amply reinforced with adhesive tape are then applied to immobilize firmly the homografted area.

All skin homografts in these studies were examined for the first time on the fifth postoperative day. Bulky compressive dressings were then reapplied and continued until the ninth or tenth postoperative day, when sutures were removed. Dressings were changed and the graft examined every 2 days following the first change of dressing on the fifth postoperative day. In some human volunteers, serial punch biopsies were removed from the host-graft junction every 3 to 5 days for microscopic study by use of a hand-operated Reese biopsy trephine, under 2 per cent local procaine anesthesia. Using a clockwise, counter-clockwise cutting motion, this trephine is employed much in

* This technique was developed by the author in collaboration with John M. Converse at the Plastic Surgery Unit, Department of Surgery, New York University College of Medicine, New York, N. Y. The photographs in FIGURES 1 to 20 were taken at the Plastic Surgery Unit when the author was a Milbank Fellow in Plastic Surgery (1951-1952).

the same manner as a simple cookie cutter. The results of these biopsies will be reported in a subsequent paper.

The usual behavior of a full-thickness skin homograft transplanted between ordinary, nonrelated individuals can be observed in the process of "initial take" on the fifth postoperative day and its gradual rejection on subsequent days in PLATE 1. The "initial take" is shown in FIGURE 1. The onset of vascular thromboses, hemorrhage, and extravasation in the graft dermis (the first signs of a homograft "rejection" reaction) are seen in FIGURE 2. Sloughing of the superficial epithelium with exposure of raw graft dermis or "dermal pad" lying underneath can be noted in FIGURE 3. The first appearance of "dry" necrosis or gangrene of the denuded graft dermis is observed in FIGURE 4 on the 14th postoperative day. This is even more apparent in FIGURE 5 on the 19th postoperative day. On the 21st postoperative day (FIGURE 6) the entire "dermal pad" is gangrenous. From the photographs taken on the 26th postoperative day to the 51st postoperative day (FIGURES 7 through 13, PLATES 2 and 3) a gradual disintegration, absorption, and sloughing of the remaining graft dermis can be noted. Within the same time period a gradual contraction of the wound edges of the full-thickness defect that eventually results in closure of the wound by a union or apposition of its original edges is evident. Billingham and Medawar²¹ noted this same contracture and intussusceptive growth in the healing of full-thickness defects in rabbits. In FIGURE 14 (PLATE 3), on the 54th postoperative day, the last vestiges of "dermal pad" have finally disappeared and epithelialization from the edges of the defect is almost complete. The usual behavior cycle of a full-thickness skin homograft transplanted between ordinary, nonrelated individuals has thus run its course from "initial take" to "dermal pad" and complete disintegration and disappearance.

Medawar's observation²² on rabbits that a second skin homograft taken from the same donor and transplanted to the same host undergoes a more rapid rejection and disintegration (the "second-set" phenomenon) is conclusively verified by transplantation of full-thickness skin homografts in human volunteers (PLATE 4). Observe the mottled, hemorrhagic, "dead" second-set graft in FIGURE 16, photographed on the 3rd postoperative day, in comparison to the more normal, pink areas of "initial take" in FIGURE 15 of the first-set graft. (These differences are even more apparent in the original Kodachrome color prints from which these black and white photographs are reproduced.) The more rapid rejection is better illustrated by comparing FIGURES 17 and 18, in which the first-set graft (FIGURE 17) on the 9th postoperative day is still two-thirds grossly uninvolved by hemorrhage and pink in appearance, whereas the second-set graft (FIGURE 18) on the 10th postoperative day is markedly hemorrhagic and already gangrenous. FIGURES 19 and 20, respectively, photographs of the first- and second-set grafts taken on the 12th postoperative day are the most dramatic in demonstrating the more rapid rejection of second-set grafts taken from the same donor and applied to the same host on the opposite thigh or arm, thus emphasizing the "systemic" nature of the homograft rejection phenomenon.

The small, round, punched-out areas seen in the photographs at the host-graft junction are areas from which biopsies were taken with the Reese trephine.

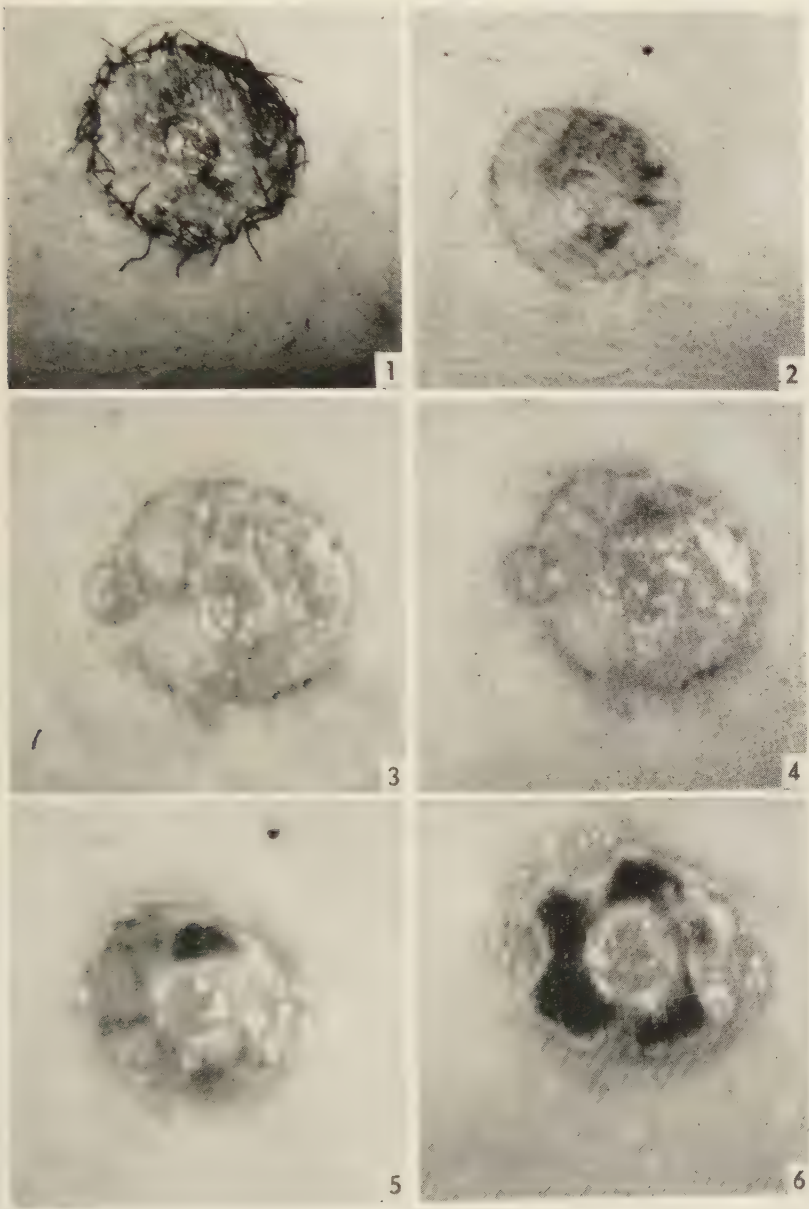


PLATE 1. *Behavior of a First-Set Homograft.*

- FIGURE 1. Fifth postoperative day: "initial take."
FIGURE 2. Ninth postoperative day: first onset of hemorrhage and epithelial desquamation.
FIGURE 3. Twelfth postoperative day: epithelial desquamation denudes the raw dermal bed.
FIGURE 4. Fourteenth postoperative day: first appearance of the dry, necrotic dermal pad at "12 o'clock."
FIGURE 5. Nineteenth postoperative day: further progression of dry gangrene in the dermal pad.
FIGURE 6. Twenty-first postoperative day: the dermal pad is entirely gangrenous.

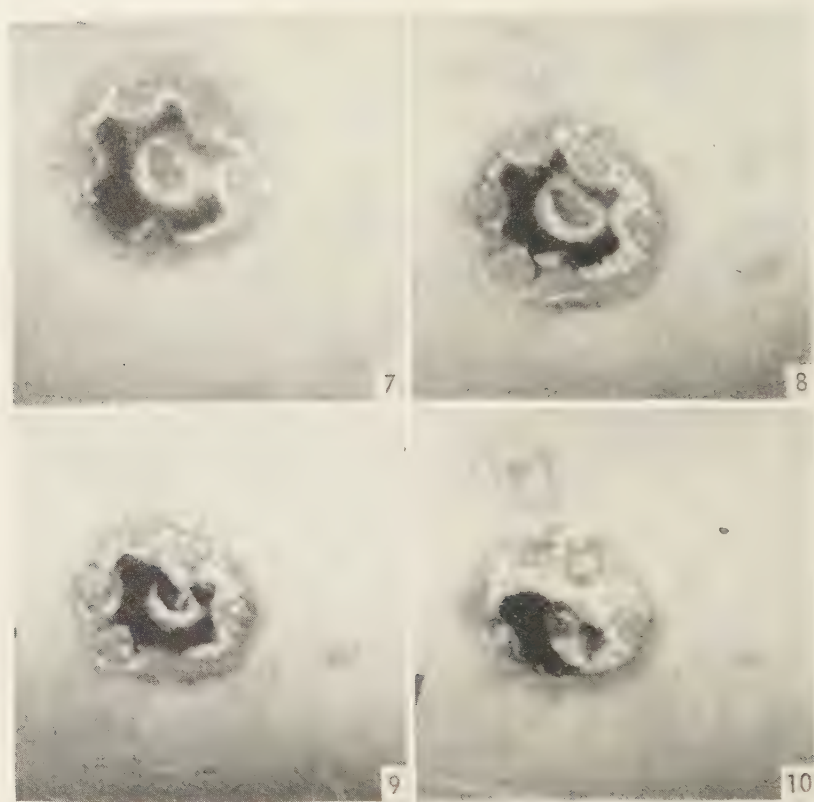


PLATE 2. *Behavior of a First-Set Homograft (Continued from Plate 1).*

FIGURE 7. Twenty-sixth postoperative day: gradual erosion of the dermal pad.

FIGURE 8. Thirty-second postoperative day: gradual erosion of the dermal pad.

FIGURE 9. Thirty-fourth postoperative day: note the contraction of the wound edges.

FIGURE 10. Thirty-eighth postoperative day: some of the dry, gangrenous dermal pad has sloughed.

We feel that this biopsy technique does not disturb the graft itself as it does not create any motion of the graft on the bed to which it has been applied. By the time the first biopsy specimen is taken on the 5th postoperative day, the graft is usually firmly anchored to its host bed by the healing processes involved in the "initial take."

With this described technique serving as a reference background to the usual behavior of full-thickness skin homografts in nonrelated humans, it is perhaps advisable at this point to mention briefly the significance of the twin studies reported in this paper from an aspect other than that of homografting.

In his extremely interesting book "*Heredity In Health And Mental Disorder*," Franz J. Kallmann²³ states: "The phenomenon of twinning does supply the human sciences with many valuable sets of genotypically identical individuals whose dissimilarities can be compared with the behavioral variations observed in ordinary sibs or two egg twins. Over one and a half million pairs of twins

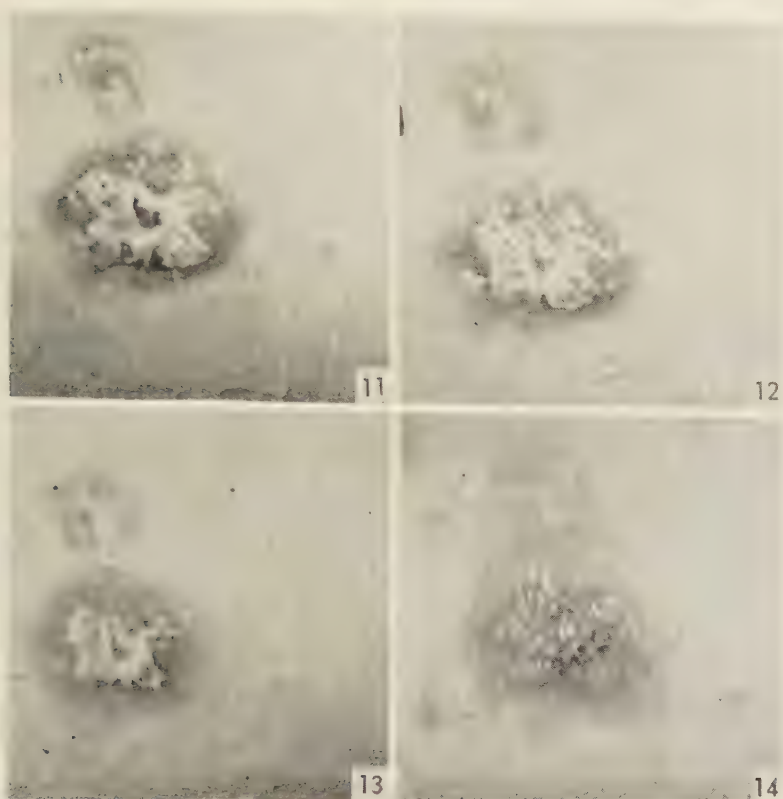


PLATE 3. *Behavior of a First-Set Homograft (Continued from Plate 2).*

- FIGURE 11. Forty-first postoperative day: the entire dry, gangrenous portion of the dermal pad has sloughed.
 FIGURE 12. Forty-eighth postoperative day: remnants of the dermal pad on the raw granulating bed.
 FIGURE 13. Fifty-first postoperative day: the contracted wound edges have almost completely cast off the dermal pad remnants.
 FIGURE 14. Fifty-fourth postoperative day: the dermal pad of the original homograft has completely sloughed.

are available in the United States for scientific studies . . . one quarter to one third of this number being of the one-egg (monozygotic) variety."

Certain mental deficiencies, such as Mongolism, have interested geneticists and psychologists alike, especially because the etiology of these disorders is obscure. When, therefore, there arises a situation in which a pair of twins is characterized by 1 normal twin and 1 Mongoloid twin (see FIGURE 21), the accurate differentiation between monozygosity and dizygosity in these twins becomes a matter of great importance to these research workers. If the Mongoloid child proves to be an identical twin, the mere existence of the physical and mental normality of his twin brother would help to discredit or make less valid the theory of hereditary Mongolism and, in turn, would add more weight to the influence of acquired or environmental etiologic factors in the development of Mongolism, especially in such a child. These same findings could apply

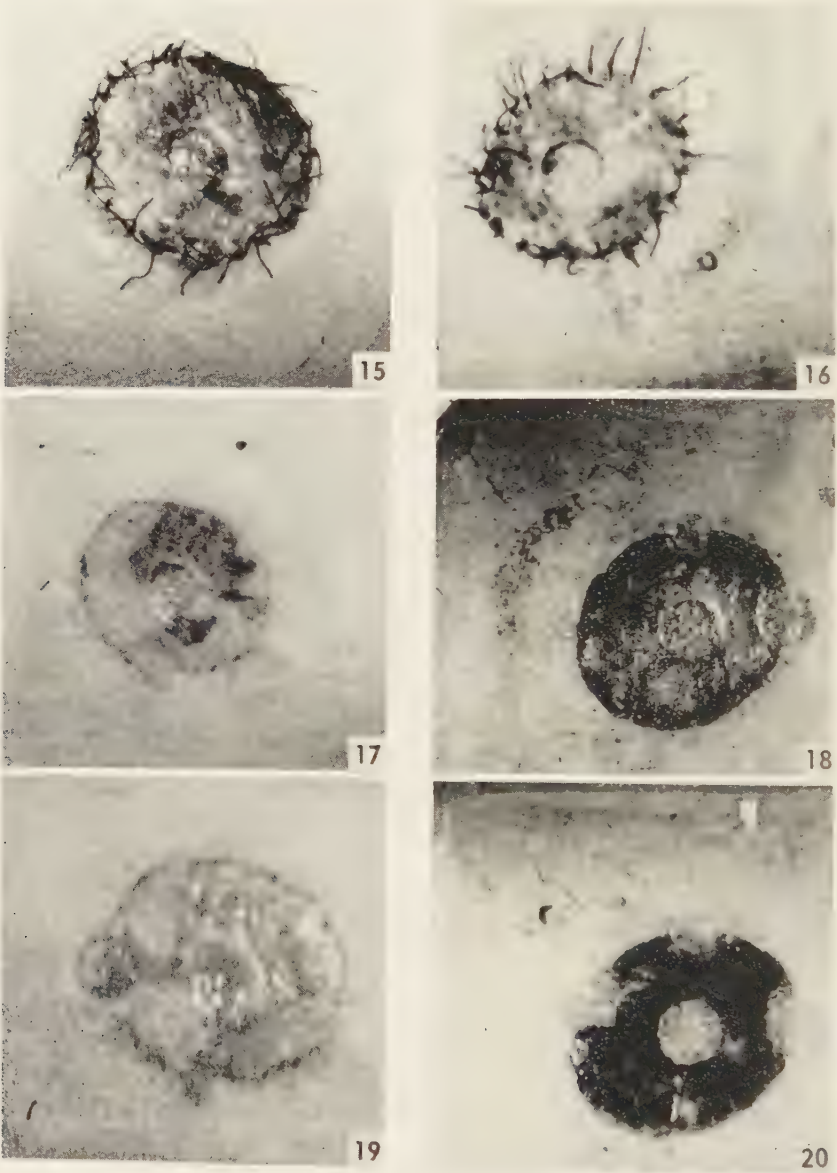


PLATE 4. *Comparison of First-Set and Second-Set Skin Homografts.*

- FIGURE 15. Fifth postoperative day: "initial take." The graft is viable.
- FIGURE 17. Ninth postoperative day: the first onset of hemorrhage and vascular thromboses. Some portions of the graft are still viable.
- FIGURE 19. Twelfth postoperative day: the epithelium is desquamating, exposing the raw dermal bed.
- FIGURE 16. Third postoperative day: despite the "initial take," the graft is severely thrombosed and hemorrhagic.
- FIGURE 18. Tenth postoperative day: the graft is dry and gangrenous. The epithelium has sloughed.
- FIGURE 20. Twelfth postoperative day: the graft is completely and intensely gangrenous.

equally well to the role of hereditary versus acquired or environmental etiologic factors in the development of a cleft lip or palate or any other congenital deformity in 1 of 2 proved "identical" twins.

Case Reports

In cases I, II, and III the zygosity of 3 pairs of twins could not be completely or conclusively determined by the usual comparative genetic tests and criteria. This quandary presented certain difficulties to the geneticists and psychiatrists who were studying mental deficiencies in twins*. As a last resort, the present skin homografting procedures were performed in the hope of determining the zygosity of each of the 3 pairs of twins. Since, to date, all reciprocal skin homografts in human identical twins have behaved as autografts do and have survived permanently, the permanent survival of homografts in any of these twins would serve at least as a diagnosis of "monozygosity."

In cases I and II, the blood antigens were identical and both pairs of twins had very similar dermatoglyphic patterns. In order to rule out any possible blood chimerism,²⁴ the blood of all 6 twins was examined at the Knickerbocker Foundation, Inc., New York, N. Y., for heterogeneity of the red blood cells in respect to the ABO antigens, with negative results. Since in each case 1 twin of a pair was severely defective, both mentally and physically, ordinary morphological criteria of zygosity could not be relied upon. In case III, the blood antigens were identical with the exception of the P group; one twin was P+ and the other P-.

In case I, that of 11-year-old twin boys, the defective member was a Mongoloid imbecile (FIGURES 21 and 22). The Mongoloid had a moderate amount of brown (superficial) eye pigmentation that was lacking in his normal brother. Their blood group antigens were identical, as follows:

Patients D.C.: A₁, M, Rh(D + C + E- c-), S + s+, Fy^a-,

R.C. K- k+, Le^a-, Jk^a+, P-

In case II, that of 13-year-old girls (FIGURES 23 and 24) the defective member had microcephaly of postnatal origin, retrolental fibroplasia, and a symmetrical growth anomaly of the toes. Their blood group antigens were identical, as follows:

Patients M.W.: A₁, MN, Rh(D+ C+ E- c-), S- s+, Fy^a+,

D.W. K- k+, Le^a-, Jk^a+, P-

In case III, A.M. and K.M. were 10-year-old twin girls, born to a 38-year-old mother as the last of her 6 pregnancies. Eight days following their delivery,

* The study of cases I, II, and III reported in this paper was part of a study of mental deficiency in twins now in progress at the Department of Medical Genetics of the New York State Psychiatric Institute, New York, N. Y. The larger project is under the joint supervision of Franz J. Kallmann and the Laboratory of Socio-Environmental Studies, Research Branch, National Institute of Mental Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md. The homograftings in cases I and II were performed in Kallmann's Department of Medical Genetics at the New York State Psychiatric Institute. Clinical studies and preliminary tests of zygosity were carried out by Gordon Allen of the National Institutes of Health, and blood group studies were made by Philip Levine of the Ortho Research Foundation, Raritan, N. J.



FIGURE 21. Case I: dizygotic twins. FIGURES 21a and 21b show the mongoloid twin, and FIGURES 21c and 21d show the normal twin

the mother's uterus was found to contain two additional fetuses, macerated but identified as male and female. A.M. was diagnosed as Mongoloid at one week of age. When tested in 1954, K.M. had a normal physical and mental development, with an I.Q. slightly above the mean of her normal sibling.



FIGURE 22. Family portrait of the case-I twins shown in FIGURE 21 (mongoloid twin at left).

A.M. was typically Mongoloid in the same year, with a flattened occiput, hypoplastic nose, crooked fifth fingers, simian creases in both palms, and wide separation between the first and second toes. At the present date, both children have straight reddish-blond hair and blue eyes, although there is a ring of yellow pigment near the pupil of K.M. that A.M. does not have. Both children have free ear lobes and median type chins. Fingerprints were compatible with a diagnosis of monozygosity. Other anthropologic-genetic calibrations suggested that the twins were monozygotic. Blood tests performed by the Knickerbocker Foundation gave similar results in all but 1 blood factor, as follows:

Patients A.M.: B, MN, Rh₂(D+ C- E+ c+ e+), S- s+, Fy^a+,

K.M. K- Le^a-, Jk^a-



FIGURE 23. Case II: monozygotic twins. FIGURES 23a and 23b show the retarded twin (microcephaly), and FIGURES 23c and 23d show the normal twin.

With respect to P, however, which had not been tested prior to the skin homografting, A.M. was positive and K.M. was negative.

Clinical Results

Full-thickness, circular skin homografts were reciprocally transplanted in corresponding defects made on the volar surface of the left forearms of each set



FIGURE 24. X rays of the symmetrical growth anomaly of the fourth and fifth toes of the case-II retarded twin shown in FIGURES 23a and 23b.

of twins. The normal "initial take" of these grafts may be observed in FIGURE 25, photographs taken on the 7th postoperative day of the homografts placed on the forearms of the normal twin in case studies I and II. Between the 19th postoperative day and the 22nd postoperative day (FIGURE 26) in the twin brothers (case I), a sudden violent rejection of the skin homografts occurred. This was evidenced by redness and induration on the 19th postoperative day, and by marked induration, redness, vascular thromboses, hemorrhage, and ulceration on the 22nd postoperative day. The grafts became gangrenous rapidly, and the entire full-thickness of skin in both twins was promptly sloughed off, leaving behind a small defect (FIGURE 27a) which, at the ninth week, after transplantation, had healed with a moderate amount of scar tissue and epithelialization from the wound defect's edges.

In the twin girls (case II), however, the homografts on the 19th postoperative day, the 22nd postoperative day (FIGURE 28), and 9 weeks after transplantation (FIGURE 27b) survived, and behaved in every respect as autografts do. Survival 1 year later indicates a probable monozygotic relationship in the twin girls. Except for the relatively delayed "rejection phenomena" in the boys (in comparison to the usual chain of events occurring on or about the seventh to the ninth postoperative day in unrelated donors and recipients, as shown in FIGURES 2 and 3), the phenomena observed were identical to those associated with the sloughing of skin homografts between 2 unrelated individuals (FIGURES



FIGURE 25. Seventh postoperative day: "initial take" of the homograft in case I (FIGURE 25a) and the homograft in case II (FIGURE 25b).



FIGURE 26. Case-I (dizygotic twins) homograft between the 19th (FIGURE 26a) and the 22nd (FIGURE 26b) postoperative day. In FIGURE 26a note the onset of induration, confined only to this homograft. FIGURE 26b shows induration, hemorrhage, and ulceration.



FIGURE 27. Case-I (FIGURE 27a) and case-II (FIGURE 27b) homograft sites in the ninth postoperative week. The case-I homograft site shows a small scarred defect where the homograft sloughed and healed by epithelialization from the wound edges. The case-II homograft is normal, healthy, and intact.

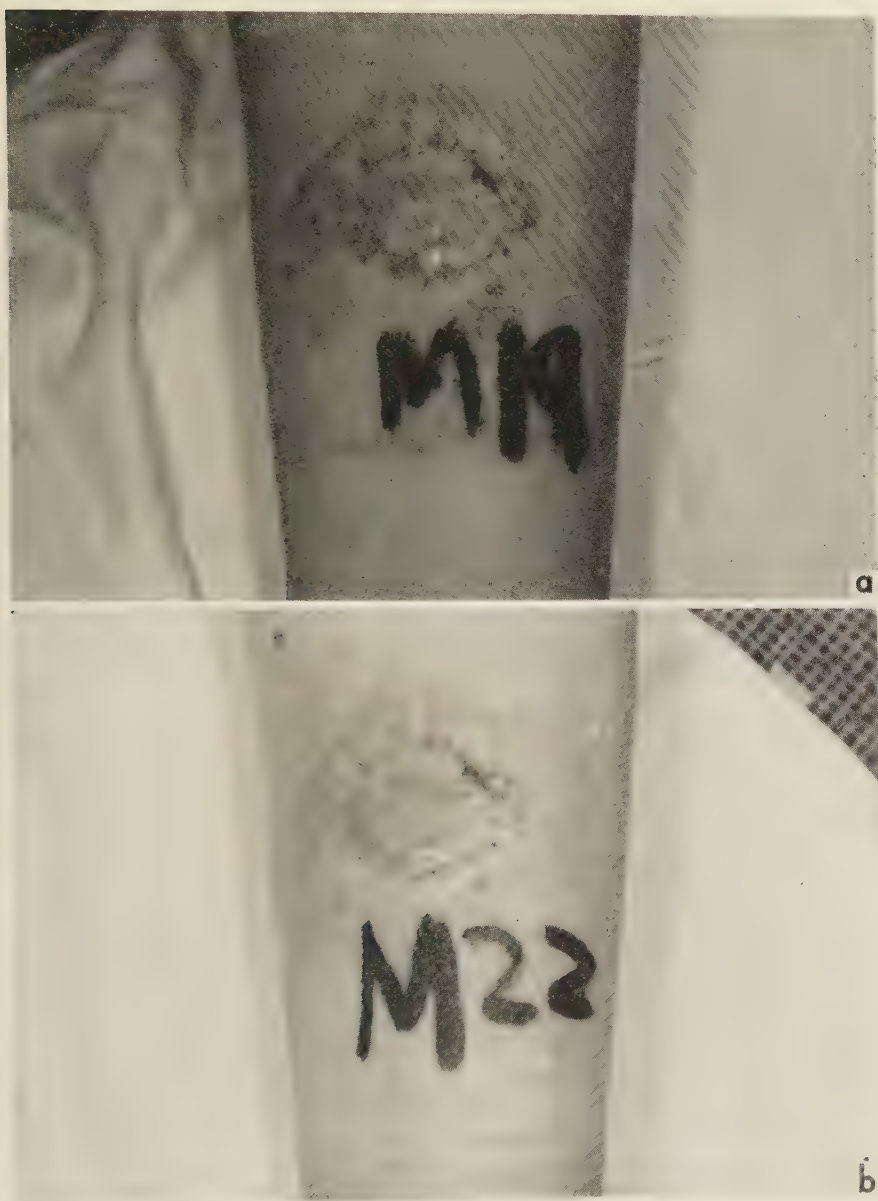


FIGURE 28. Normal, healthy appearance of the case-II (monozygotic twins) homograft between the 19th (FIGURE 28a) and the 22nd (FIGURE 28b) postoperative day.



FIGURE 29. Case IV (monozygotic twins). The normal twin is on the left. The twin on the right has a cleft palate and fusion deformities of the cervical vertebrae.

1 to 14). The sloughing of reciprocal homografts in both boys, following a long "initial take" of almost 3 week's duration, is not typical of the behavior of any successful autograft.

In case III,* follow-up examinations made in the weeks subsequent to cross-homotransplantation revealed grafts that remained essentially normal, appearing in all respects like skin autografts until the 22nd postoperative day. At this time, the graft of K.M., the normal girl, suddenly took on a reddened, indurated, edematous appearance and, within a few days, became rapidly ulcerated and necrotic. Approximately 1 week later, the same destructive chain of events that lead rapidly to sloughing of the graft also took place in A.M., the Mongoloid girl. These delayed rejection phenomena were identical to those described in case I and those seen in the sloughing of skin homografts between 2 unrelated individuals. The results in cases I and III, therefore, were taken as conclusive evidence that the twins in each case were indeed dizygotic in origin.

An additional case IV presented us with the opportunity of transplanting reciprocal skin homografts between little twin girls†, one of whom had a cleft

* Reuven K. Snyderman of the Sloan-Kettering Institute for Cancer Research, Memorial Center, New York, N. Y., generously served as cosurgeon in the reciprocal transplantation procedures performed in case III.

† This case was obtained through the kind efforts of John C. Walker, St. Barnabas Hospital, Newark, N. J., who also served as cosurgeon in the reciprocal transplantation procedure.

palate and fusion deformities of her cervical vertebrae (FIGURE 29). The mother was convinced that these children were dizygotic or nonidentical. Skin homografting was employed, not only to help the mother settle in her own mind the zygosity of her twins, but also to show that it is a feasible procedure to use in studying the role of heredity versus acquired or environmental etiologic factors in the development of cleft lip and cleft palate.

Careful follow-up examinations revealed the normal appearance of these grafts for the 5 weeks immediately following the homografting procedure. Because of the age of these children and the cosmetic or aesthetic preferences of the mother, the full-thickness circular skin homografts were transplanted to the medial surface of the left arm rather than to the volar aspect of the left forearm, as in cases I, II, and III. These grafts were inspected 1 year later; they have continued to survive and show no signs of sloughing. We have taken this as conclusive evidence that these little girls, as well as the twin girls of case II, are monozygotic or identical twins. It was also concluded from case IV that acquired or environmental factors played a more prominent role in the development of cleft palate in one of these twins than did hereditary factors.

Discussion

There seems to be little room for doubt, on the basis of the available medical literature, that full-thickness skin homografts, reciprocally transplanted, survive permanently in identical (monozygotic or 1-egg) twins. Cases II and IV in this paper are two additional pieces of clinical evidence confirming this biologic fact.

Cases I and III seem to provide clinical evidence that full-thickness skin homografts, reciprocally transplanted, are rejected and sloughed off by non-identical (dizygotic or 2-egg) twins. An additional case recently reported by Bishop²⁹ seems to confirm these findings. Whereas cases I and III in this study were conducted as elective experimental trials with skin homografts and graft-beds of a known uniform size, Bishop was confronted with an emergency clinical problem entirely. He had the opportunity to homograft 2 female twins, aged 2½ years, who were both burned by hot water and treated by identical methods. The girls "looked very alike" and were believed to be identical twins by the mother, because they were supposedly delivered with 1 "afterbirth."

One of the twins, more extensively burned than her sister, was considered unfit for autografting. Because the twins were believed to be probable monozygotics, excess skin was taken from the less extensively burned twin and cross-transplanted to her sister. On the 10th postoperative day, the appearance of the skin homografts was not unlike that of autografts, and the little girls were still considered to be identical. Between the 23rd and 26th postoperative day, however, the grafts suddenly disintegrated. Blood group studies made of the twins had become available to the surgeon by this time, and it was apparent that the twins were, in fact, nonidentical. The family doctor was contacted during this same period, and he informed the surgeons that the twins had actually been delivered with 2 placentae that were joined together by connective

tissue. Their blood groups were recorded as follows:

Janet: O, N, CDe/ cDE (R_1R_2), S+, Fy^a- , K+

Mary: O, M, CDe/ cDE (R_1R_2), S+, Fy^a+ , K-

Janet's serum showed "... no agglutination with Mary's cells at 20° or 37° C. in saline or by indirect Coombs' test. No antibodies detected against a panel of cells to include the Rh, MNS, Lu, P, Le, K, Fy^a , or Jk^a blood group systems." Bishop concluded: "Obviously, close blood group relationship has no influence on the final survival of homografts, but may be the reason for the prolonged survival time."²⁵

The previously mentioned cases and the clinical and experimental results that they produced are supported by similar observations in lower animals.

Skin homografts exchanged between dizygotic twin sheep²⁶ or ordinary cattle siblings²⁷ are rejected. This is in contrast, however, to the fact that skin homografts between some dizygotic twin cattle frequently survive indefinitely.²⁷ These dizygotic twin cattle, on the other hand, represent a distinct genetic entity not generally encountered in humans or sheep; this entity is the condition known as freemartin twinning. The contrast between cattle and sheep in this respect has been attributed^{27, 28} to the interchange of fetal blood in cattle twins, as evidenced by the occurrence of freemartins in cattle. Furthermore, each member of these dizygotic twin pairs of cattle usually has 2 types of red blood cells,²⁹ a heterogeneity suggestive of an actual transplantation of blood-forming tissues by way of the fetal or vascular anastomoses. Anderson *et al.*²⁷ suggest "that the anomalous tolerance of dizygotic cattle twins to grafts of each other's skin has the same origin as their conformity of immunological blood types." Such animals are spoken of as erythrocyte chimeras. At least one such chimera has been described in man.²⁴

In the cases reported in this study, there are those who might question the advisability of resorting to skin homografting when blood grouping, fingerprinting, or other tests can be performed more easily. It should be remembered, however, that wide differences in fingerprints can occur in monozygotic twins.³⁰ Recent studies by Osborne³¹ have shown that among same-sex twins having the same blood group factors, approximately 15 to 18 per cent may be dizygotic. This finding cancels out the absolute accuracy of using identical blood groups as a test for monozygosity. In his 1955 Ph.D. thesis at Columbia University³¹ Osborne finally diagnosed 34 same-sex pairs as dizygotic in a study of 100 pairs of adult twins. Of these 34 pairs, 6 (or 18 per cent) showed no difference with respect to reliable blood group factors including ABO, Rh(5 factors), MNS, Kell, Duffy, and others. Allen³⁰ emphasizes that fingerprinting, anthropologic measurements, and other procedures will give rather good genetic evidence for identifying the dizygosity of some of the 15 to 18 per cent of dizygotic twins with identical blood group factors, "but there is bound to be a residue that can be recognized only by skin homografting."

The previously mentioned clinical findings certainly suggest that more than a casual role is played by inherited genes in the behavior of skin homografts in humans. Employing the technique outlined earlier in this paper, in homo-

grafting 8 different unrelated donors and recipients the author observed that the first gross signs of homograft rejection began on approximately the seventh postoperative day. Converse and Rapaport,³² improving upon this original technique by use of stereomicroscopic methods in an additional 15 different unrelated donors and recipients, also observed that the first microscopic signs of homograft rejection (cessation of blood flow in graft vessels) ranged from the 6th to the 10th postoperative day, with a mean survival time of 8.09 days.

When the genetic relationship of donor and recipient, however, is more closely allied (familial), as in cases I, II, III, and IV, it becomes obvious that the mean survival time based upon the first signs of graft rejection (vascular thromboses, hemorrhage, or frank ulceration) is considerably prolonged. In the 4 dizygotic twins described in this study, the first gross signs of homograft rejection ranged from the 19th to the 29th postoperative day. These figures are probably more significant because they represent human experiments in which the size of the homograft and the graft defect were accurately controlled. None of these patients received any drug or medication other than routine antibiotic therapy—for example, penicillin. At no time did any of the human volunteers or twins in this report, or in that of Converse and Rapaport, receive antihistamines, cortisone, or cortisonelike preparations.

This is in contrast to the lack of controls in clinical reports in the literature in which prolonged survival (3 to 6 weeks) of some skin homografts is reported in critically burned patients, even when the homografts are donated by genetically unrelated individuals. The latter clinical reports, however, seem to have little significance in any controlled study of a possible direct relationship between genetic similarity of donor and recipient and an increased length of survival time of the donor's skin homografts dependent upon the closeness of that similarity. Homografts from unrelated donors occasionally survive longer in critically burned patients, probably for the following reasons:

(1) The natural stress response of the adrenals is surprisingly large in critically burned patients.³³ Also, the more severely burned the individual, the more pronounced is his tendency to show greater excretion of urinary corticosteroids and higher blood levels of corticoids.³⁴ This increased corticosteroid activity in burned humans is analogous to the studies of Billingham *et al.*³⁵⁻³⁶ and of numerous more recent investigators who have amply demonstrated that skin homograft survival time can be significantly prolonged by administration of cortisone or other corticosteroids.

(2) To a lesser extent, Halsted's law³⁷ [or a modification thereof as applicable to skin grafting] may also possibly play a role in prolonging the life of skin homografts obtained from unrelated donors in burned patients. Halsted postulated that an endocrine [or skin] graft is more likely to be successful [or have its life prolonged temporarily] if a deficiency [or defect] exists in the host of its particular secretion [or function]. (Words in brackets are the author's.)

Increased corticosteroid activity and, possibly, a modification of Halsted's law therefore probably explain the prolonged survival in burned patients of some skin homografts obtained from unrelated donors. This contrasts with the short 7- to 9-day average survival time of equivalent skin homografts in controlled human experiments.

We must therefore consider the 2 sets of controlled findings reported in this study, which suggest the practicability of using a genetically similar donor in preference to an unrelated donor for skin homotransplantation in the critically burned patient. Whereas skin homografts survive for 7 to 9 days in unrelated donors and recipients, they survive for 19 to 29 days in closely related donors and recipients (as in cases I and III). Additional studies by the author are now under progress to determine the average survival time of skin homografts transplanted between brothers, sisters, parents, grandparents, and newborn infants, employing the controlled techniques already described. It is hoped that there will result from these studies some practical law of genetic relationship that may have a direct bearing upon the choice of "ideal" donor material in any future skin homografting in the human. Our results to date compare favorably with those of Schoene³⁸ and Loeb,³⁹ at least from the standpoint of suggesting a possible genetic gradation similar to that proposed by Loeb.³⁹ "The average genetic relationship between near relatives, such as brothers and sisters, parents and children, should be somewhere intermediate between the homoïgenous (nonrelated) and autogenous relationship, and, accordingly, the average results of syngenesiotransplantation (related) should likewise be somewhere intermediate between those of autogenous and homoïgenous transplantation."

Schoene obtained his most satisfactory results when transplanting in rats between brothers and sisters.^{38a, b} In random syngenesiotransplantations between members of noninbred families of guinea pigs, the best survival times were obtained by Loeb³⁹ in brother-to-brother (sister) transplantations, whereas there was little significant difference in the slightly shorter survival time when tissues were reciprocally transplanted from parents to children, or from children to parents. To date, clinical reports in the literature of human homotransplantation also record the longest survival times when familial-related donor-recipient transplantations (syngenesiotransplantation) are carried out. Permanent survivals have been described in recent years by Wolf⁴⁰ (donor, father; recipient, son); Kearns and Reid⁴¹ (donors, mother and father; recipient, son); Caby⁴² (donor, mother; recipient, daughter); and Meek⁴³ (donor, father; recipient, son).

It is worthy of note that, within this same span of years, there has been recorded no case of a permanent survival of nonrelated donor-recipient homografts. Of equal interest is the finding that in 2 of the forementioned cases^{41, 43} the recipient had received transfusions of whole blood from his parent donors. The possible significance of these blood transfusions in determining the outcome of the skin homografts is discussed by Snell,⁴⁴ who believes that in the case of Kearns and Reid⁴¹ "it is interesting to inquire whether . . . successful homotransplantation of skin may not have been due to the prior blood transfusions and, hence, still another parallel of the enhanced growth of tumor and normal tissue transplants produced by prior injections of lyophilized tissue."

Regarding the effect of blood group antigens on the ultimate outcome of skin homografting, Pfeffer and Rogers⁴⁵ report a preliminary study of the behavior of skin homografts in rats with compatible and incompatible blood groups. When compatibility of blood groups of donor and host was present, skin homo-

grafts transplanted from the donor to the host were rejected less violently than grafts transplanted between a donor and host whose blood groups were incompatible. This suggests that factors connected or identical with blood group factors may play a role in determining the ultimate behavior of skin homografts. Certain specific blood group antigens responsible for homograft rejection may, in fact, be identical with skin antigens. It is advisable to remember that in the broader approach to this problem, blood groups as well as skin antigens are both genetically determined. Here again, the importance of gene-controlled factors is emphasized; it may very well be that the effects of a gross genetic similarity of skin antigens in donor and recipient can be "reinforced" by the effects of a gross genetic similarity of blood group antigens, with the result that the survival time of a skin homograft between a closely related human donor and a recipient with compatible blood groups is prolonged even longer than it would be if only the skin antigen similarity or blood group antigen similarity were acting alone rather than in combination. This is suggested by the somewhat longer survival time of control reciprocal skin homografts fitted into control skin defects, the donor and recipient of these homografts having been chosen from the general population by Woodruff and Allan⁴⁶ because of their same blood group combination. Neither donor nor recipient was genetically related! Their blood group combination, however, was identical for the following groups [O, MN, Rh (cde cde), S—, Duffy+, K—, Le^a— b⁺, Lu—]. In contrast to the average survival time of 7 to 9 days noted in our nonrelated donors (FIGURES 1 to 14), the survival time of the grafts in 1 of these 2 donors varied from 14 to 21 days.

The possible "reinforcement effect" of skin antigen similarity based on a close genetic relationship and blood group antigen similarity in bringing about a markedly prolonged survival of skin homografts taken from a 27-year-old donor and placed upon his 35-year-old severely burned brother is suggested in the case described by Kay⁴⁷ in this monograph. These homografts survived and proliferated for 32 weeks before undergoing rapid disintegration. Although the patient was placed on cortisone therapy for the first 6 months of his hospitalization, the actual effect of this therapy on the survival of these homografts is questionable, since it did not seem to have any significant effect in increasing the survival time of skin taken from 17 other donors, 9 of whom were his brothers or sisters and the remainder more distantly or not at all related.

A detailed blood grouping of the two brothers revealed the following:

Patient: A₁, N, Rh (cde cde), Fy^a—, K—, Le^a—b[—], Lu^a—

Donor: A₂, M, Rh (cde cde), Fy^a—, K—, Le^a—b⁺, Lu^a—

In case I of this study, however, it is also apparent that absolute blood group antigen similarity in the nonidentical twins was not necessarily sufficient protection against the ultimate rejection of skin homografts that took place between the 19th and 22nd postoperative day. One might hypothesize that the skin antigen dissimilarity played a more prominent role in the rejection phenomena observed here. Likewise, in case III a marked compatibility between the blood group antigens of both nonidentical twins was also insufficient to prolong

the survival time of the homografts beyond a 22 to 29 postoperative-day survival period.

Woodruff and Allan⁴⁶ probably offer the most succinct and yet speculative conclusion, which applies equally well to cases I and III in this report, as well as to the others cited. Commenting upon the rejection of skin homografts between their nonrelated donor and recipient with identical blood group patterns, they state: "It is almost certain . . . that our two volunteers differed in respect of some red-cell antigens not yet investigated, and the possibility that these unknowns were identical with the skin antigens responsible for the breakdown of the grafts cannot be entirely excluded." Their suggestion, that more complex and as yet unknown or undetected red cell antigens may be identical with skin antigens responsible for homograft rejection, provides us with yet another hypothesis, in which the possible identical nature of blood and skin antigens is contrasted with separate and distinct skin antigens and blood group antigens acting singly, or reinforcing the effects of each other in combination, as suggested above. Further detailed and controlled studies of reciprocal skin homotransplantation in related and nonrelated human donors, coupled with further refinements in identifying additional possible blood group antigen systems, will probably help to clarify or refute one or another of the hypotheses suggested above. The previously mentioned findings, however, suggest the following interpretations:

(1) *When skin homografts are obtained from a closely related human donor ("syngeneisotransplantation"), their temporary survival time on the recipient under control conditions is longer than skin homografts obtained from nonrelated donors.*

(2) *When gross compatibility of all the major and minor blood groups and subgroups known to date exists between a donor and recipient, skin homografts transplanted under control conditions between this donor and recipient survive longer than skin homografts obtained from donors with dissimilar or incompatible blood groups and subgroups.*

In the light of these studies, it may also be assumed that extremely delicate and refined blood-typing techniques might possibly result in the identification of new or as yet poorly demonstrable blood subgroups, thus serving as a method of "typing" other tissues, especially skin. Simonsen⁴⁷ suggests that one "must aim at thorough congruity of the blood group combinations in donor and recipient. This is a complicated task, which, however, is not impossible to solve. If the result proves negative, we still do not know whether this is because blood groups play no part in skin homografting, or whether there are still undiscovered blood groups of equally great or even greater importance than those already known."

Summary

(1) Full-thickness reciprocal skin homografts are rejected by dizygotic human twins.

(2) Full-thickness reciprocal skin homografts survive permanently in monozygotic human twins.

(3) Full-thickness reciprocal skin homografts transplanted under control

conditions in nonrelated human volunteers chosen at random survive only for 7 to 9 days postoperatively.

(4) Full-thickness reciprocal skin homografts transplanted under control conditions in two sets of dizygotic human twins survive for periods ranging from 19 to 29 days postoperatively.

(5) When skin homografts are obtained from a closely related human donor ("syngenesiotransplantation"), their temporary survival time on the recipient under control conditions is longer than skin homografts obtained from non-related donors.

(6) When gross compatibility of all the major and minor blood groups and subgroups known to date exists between a human donor and recipient, skin homografts transplanted under control conditions between this donor and recipient survive longer than skin homografts obtained from donors with dissimilar or incompatible blood groups and subgroups.

(7) In treating the severely burned patient, when skin homografting is indicated, donor graft material should preferably be taken from closely-related members of the patient's own family whose blood groups are as similar as possible to the patient's own blood groups. This should help to guarantee a significantly longer temporary survival of the homografts, in contrast to the shorter survival time of grafts taken from nonrelated donors.

(8) Preoperative transfusions of compatible blood from parents, brothers, or sisters, possibly combined with subsequent postoperative transfusions of the same blood, may serve to enhance the survival of subsequently transplanted skin homografts taken from the same parents, brothers, or sisters. This mechanism may be similar or identical to the "enhancing effect" of Snell and others.

References

1. ROGERS, B. O. 1951. *Plastic & Reconstr. Surg.* **7**: 169.
2. ROGERS, B. O. 1954. *Transplantation Bull.* **1**: 58.
3. ROGERS, B. O. 1955. *Transplantation Bull.* **2**: 29.
4. ROGERS, B. O. 1956. *Transplantation Bull.* **3**: 19.
5. ROGERS, B. O. & G. ALLEN. 1955. *Science*. **122**: 158.
6. ROGERS, B. O. & G. ALLEN. 1955. *Transplantation Bull.* **2**: 100.
7. BAUER, K. H. 1927. *Bruns' Beitr. klin. Chir.* **141**: 442.
8. BORST & ENDERLEN. 1909. *Langenbecks Arch. klin. Chir.* **99**: 54.
9. LEXER, E. 1911. *Langenbecks Arch. klin. Chir.* **95**: 827.
10. LEXER, E. 1919. *Die freien Transplantationen*. Verlag von Ferdinand Enke. **I**. Stuttgart, Germany.
11. PERTHES, G. 1917. *Zentr. Chir.* **44**: 426.
12. EDEN, R. 1922. *Deut. med. Wochschr.* **48**: 85.
13. BILLINGHAM, R. E., G. H. LAMPKIN, P. B. MEDAWAR & H. L. WILLIAMS. 1952. *Heredity*. **6**: 201.
14. PADGETT, E. C. 1932. *Southern Med. J.* **25**: 895.
15. BROWN, J. B. 1937. *Surgery*. **1**: 558.
16. SCHATNER, A. 1944. *Arch. Otolaryngol.* **39**: 521.
17. CONVERSE, J. M. & DUCHET, G. 1947. *Plastic & Reconstr. Surg.* **2**: 342.
18. MCINDOE, A. & A. FRANCESCHETTI. 1950. *Brit. J. Plastic Surg.* **2**: 283.
19. BLANDFORD, S. E., JR. & F. A. GARCIA. 1953. *Plastic & Reconstr. Surg.* **11**: 31.
20. MEYER-BURGDORFF. 1931. *Zentr. Chir.* **58**: 1337.
21. BILLINGHAM, R. E. & P. B. MEDAWAR. 1955. *J. Anat.* **89**: 414.
22. MEDAWAR, P. B. 1944. *J. Anat.* **78**: 176.
23. KALLMANN, F. J. 1953. *Heredity in Health and Mental Disorder*. W. W. Norton. New York, N. Y.

24. DUNSFORD, I., C. C. BOWLEY, A. M. HUTCHISON, J. S. THOMPSON, R. SANGER & R. R. RACE. 1953. *Brit. Med. J.* **II**: 81.
25. BISHOP, B. W. F. 1955. *Brit. J. Plastic Surg.* **8**: 147.
26. LAMPKIN, G. H. 1953. *Nature*. **171**: 975.
27. ANDERSON, D., R. E. BILLINGHAM, G. H. LAMPKIN & P. B. MEDAWAR. 1951. *Heredity*, **5**: 379.
28. BILLINGHAM, R. E., G. H. LAMPKIN, P. B. MEDAWAR & H. LI. WILLIAMS. 1952. *Heredity*. **6**: 201.
29. OWEN, R. D. 1945. *Science*. **102**: 400.
30. ALLEN, G. 1955. Personal communication.
31. OSBORNE, R. 1955. *Heredity and Environmental Factors in Body Build: A Study Of 100 Pairs Of Adult Twins*. Thesis. Columbia Univ. New York, N. Y.
32. CONVERSE, J. M. & F. T. RAPAPORT. 1956. *Ann. Surg.* **143**: 306.
33. EVANS, E. I. & W. J. H. BUTTERFIELD. 1951. *Ann. Surg.* **134**: 588.
34. HUME, D. M., D. H. NELSON & D. W. MILLER. 1956. *Ann. Surg.* **143**: 316.
35. BILLINGHAM, R. E., P. L. KROHN & P. B. MEDAWAR. 1951. *Brit. Med. J.* **I**: 1157.
36. BILLINGHAM, R. E., P. L. KROHN & P. B. MEDAWAR. 1951. *Brit. Med. J.* **II**: 1049.
37. HALSTED, W. S. 1909. *J. Exptl. Med.* **11**: 175.
- 38a. SCHOENE, G. 1912. *Die Heteroplastische und Homoplastische Transplantationen*. J. Springer. Berlin, Germany.
- 38b. SCHOENE, G. 1916. *Bruns' Beitr. klin. Chir.* **99**: 233.
39. LOEB, L. 1945. *The Biological Basis of Individuality*. C. C. Thomas. Springfield, Ill.
40. WOLF, F. 1946. *Med. Klin. Munich.* **41**: 350.
41. KEARNS, J. E. & S. E. REID. 1949. *Plastic & Reconstr. Surg.* **4**: 502.
42. CABY, F. 1952. *Plastic & Reconstr. Surg.* **10**: 14.
43. MEEK, C. P. 1954. *Am. J. Surg.* **88**: 504.
44. SNELL, G. 1952. *Cancer Research*. **12**: 543.
45. PFEFFER, A. Z. & B. O. ROGERS. 1955. *Plastic & Reconstr. Surg.* **15**: 459.
46. WOODRUFF, M. F. A. & T. M. ALLAN. 1953. *Brit. J. Plastic Surg.* **5**: 238.
47. KAY, G. D. 1957. *Ann. N. Y. Acad. Sci.* **64**(5): 767.
48. SIMONSEN, M. 1953. *Acta Pathol. Microbiol. Scand.* **32**: 36.

PROLONGED SURVIVAL OF A SKIN HOMOGRAFT IN A PATIENT WITH VERY EXTENSIVE BURNS

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Before presenting a case report of the survival of a skin homograft for almost 8 months, I should like to make the point that, as a general surgeon, my experience with the intricacies of homotransplant research and immunology is, of necessity, limited and recent. I ask your forbearance, therefore, should any of my observations seem rash or inflammatory.

On April 8, 1954, Joseph, a patient aged 35, was admitted to Deep River Hospital, Deep River, Ont., with thermal burns involving 80 per cent of his body surface. Total skin loss amounted to 60 per cent of his body surface (FIGURES 1 and 2). Eighteen and one-half months later, completely re-covered with his own skin, he was transferred to Ottawa for further rehabilitation and occupational therapy. A full account of this case has been published in the January 1, 1956, issue of the *Canadian Medical Association Journal*.

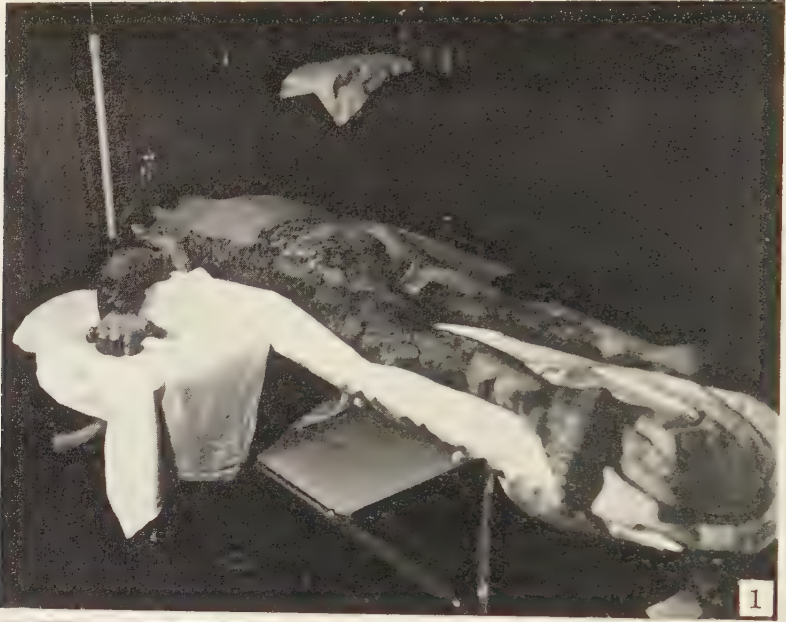
In the course of homografting, 38 drums of split skin were taken from 19 donors. Ten of the donors were his brothers or sisters, and the rest were more distantly related or unrelated. Unfortunately, Joseph was not a homozygous twin. (Neither was he uremic nor agammaglobulinemic.) The first donor's skin did not take at all, as the recipient beds were not clean enough. The result of the second donation is presented here. The other 17 donations took and survived for the expected time, the usual duration of adequate coverage ranging from 3 to 6 weeks.

On May 6, one month after admission, 2 drums of skin were taken from a brother aged 27. As mentioned, this donor was the second in chronological order. His skin was placed on three separate recipient areas—left posterior arm, left posterior thigh, and right posterior calf, as it was necessary at all times to locate both homografts and autografts in the areas best prepared to receive them. In all three sites the grafts survived and, indeed, proliferated for 32 weeks, after which rapid disintegration occurred.

During this time the patient was given continuous antibiotic therapy that varied with the results of frequent bacteriological cultures. Testosterone was given regularly, and cortisone had been administered during the first six months of hospitalization, following the advice of J. S. L. Browne of Montreal, P. Q., Canada. Cortisone, however, did not appear to increase significantly the survival time of the other homografts.

Until these apparently permanent grafts were finally rejected, it was thought that they had been replaced by a creeping substitution of the patient's own skin from surviving epithelial elements of skin glands or hair follicles. I think that FIGURES 3 to 10 show this hypothesis to be incorrect.

To investigate this phenomenon, detailed blood grouping of patient and donor was undertaken by B. P. L. Moore of the Canadian Red Cross Blood Transfusion Service, Toronto, Ont., Canada. Even before interpretation, the



FIGURES 1 and 2. April 1954. The extent of the initial problem is shown posteriorly and anteriorly. The upper back is completely denuded of skin.



FIGURE 3. May 1954. Left thigh and right calf. Two of the three areas concerned are shown one week after grafting. The other grafts seen were taken from a separate donor.

FIGURE 4. May 1954. On the left arm, just above the elbow, is the third area concerned. This graft was inadvertently cut thicker than the others, but survived just as well and as long.



FIGURE 5. July 1954. Two months after operation the grafts are still in good condition. On the thigh especially, proliferation of the edges is well demonstrated.

results are obviously similar:

Patient A_1 N cde/cde Lu(a-) K- Le(a-b-) Fy(a-)

Donor A_2 M cde/cde Lu(a-) K- Le(a-b+) Fy(a-)

1. Groups A_1 and A_2 rarely contain anti- A_2 or anti- A_1 in their respective sera. That these two sera did not contain such agglutinins was borne out by cross matching. Hence, in this case, A_1 is quite compatible with A_2 .

2. M and N antigens are relatively weak in man, and in any case occur naturally only rarely.

3. The Rh genotypes are identical, both groups being Rh -ve.

4. Both groups are Lutheran -ve, as are 92 per cent of the population.

5. Both groups are Kell -ve, as are 90 per cent of the population.

6. Like 80 per cent of the population, both groups are Lewis -ve. In this case, however, anti-Le^b in Joseph's serum was held probably responsible for an incompatibility of the donor's cells with Joseph's serum at 4° C. The clinical significance of this in the graft survival is not known.

7. Both groups are Duffy -ve. I have not been able to find the percentage distribution of this group*.

* The figures I have quoted are from *Standard Values in Blood*, Ed. E. C. Albritton, 1955; W. B. Saunders Co., Philadelphia, Pa



FIGURES 6 and 7. August 1954. Three months after operation. Continued integrity is present.



8



9

FIGURES 8 and 9. October 1954. Five and one-half months after operation. Continuity with patient's own skin is easily seen.



FIGURE 10. December 1954. Early disintegration after almost 8 months can be seen.

G. Borduas, Institute of Microbiology, University of Montreal, P. Q., Canada, was good enough to carry out recent electrophoretic studies of these men. He showed that the gamma-globulin level in the patient's serum was actually a little increased. The donor's serum showed a normal fractionation.

I now mention a most peculiar and, for me, frightening circumstance brought to light only recently by the results of this detailed grouping. When Joseph was admitted, our laboratory reported him as A Rh +ve. All blood transfusions, amounting to more than 40 bottles, were therefore A Rh +ve and, since the patient had only one minor reaction, at no time did I have cause to question the typing of his blood. As I have just stated, however, he is actually Rh -ve. Despite this, he does not, even now, show any evidence of Rh antibody formation. I understand that such a phenomenon, both in typing and lack of reaction, is unusual but not unknown.

It is unwise to draw conclusions on the basis of one experimentally uncontrolled case. At most, a demonstration of certain features may be made, and it has been shown, I think, that a skin homograft may survive for a number of months. One might deduce that in this case the homograft survival was not due to such extrinsic factors as drugs, as other homografts were rejected after the usual time. Certain other features seem worthy of note: (1) the closely similar blood groups; (2) the failure of cortisone to aid graft survival significantly, a finding previously reported by others; (3) the absence of Rh

antibodies and its possible relationship to the antigen-antibody mechanism of the active immunity hypothesis of homograft survival; and (4) the equally successful growth of the graft in three different areas.

The full interpretation of these and other aspects must probably await further research, which I sincerely hope will have been aided by this presentation. In the meantime, further work on this case may shed light on some of its obscurities.

Acknowledgment

No presentation of this case could be complete without mention of Hamilton Baxter of Montreal, whose guidance, advice, and enthusiasm were inspiring at all times.

SCALE HOMOTRANSPLANTATION IN GOLDFISH (*CARASSIUS AURATUS*)

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Introduction

There have been few detailed studies of homotransplantation reactions in adult cold-blooded vertebrates. Most of the early investigations of the integument in Amphibia were concerned mainly with the local specificity of pigmentation rather than tissue incompatibilities. Cole (1922) found that homotransplants of skin in frog tadpoles one to two years old preserve their individuality only temporarily, their tissues ultimately being replaced by regenerated tissue of the host. He considered the inflammatory reaction and disintegration of homografts as evidence that protoplasm of one individual is chemically different from that of a second individual of the same species. The definitive work of May (1923-1924) on lizards revealed that autotransplantation of skin on chameleon adults succeeded permanently, whereas all homotransplants were absorbed after they had healed in, the total absorption taking place between 60 and 90 days with the temperature kept constant at 23.5° C. On the other hand, Collins and Adolph (1926) did not observe any difference between the results of autotransplantation and homotransplantation of skin in the adult salamander. It appeared that both remained preserved, but that in both a reorganization of pigmentation took place. Later Vogel (1940) investigated in detail the fate of skin transplants in adult frogs. He found that after a latent period of about 1 week, homografts were invariably invaded by host leukocytes. Graft tissues were then destroyed and replaced by host tissues.

Goodrich and Nichols (1933) first established the existence in fish of "individuality differentials." They found that autotransplants of scales on all types of goldfish succeeded without loss of any of the tissue elements, epithelium, dermis, or fibrillary plate. But some degree of tissue incompatibility was observed in all homotransplants. This varied from that apparently effecting only the disintegration of chromatophores to that bringing about the destruction of the whole scale and accompanied by inflammation. Later Sauter (1934) and Nardi (1935), working with various fishes, found that homotransplants comprising scales, muscle, and ribs completely degenerated, while autotransplants succeeded. None of the investigators mentioned recognized the immunological basis of homograft destruction. The chief difficulties were lack of adequate criteria for the homograft survival end point and failure to appreciate the importance of temperature controls in using poikilothermic animals.

The present study is concerned with the quantitative aspects of scale homograft reactions in the goldfish. Goldfish scales are highly vascularized structures with a complex histology that has been well described by Neave (1940). Their location and mode of attachment greatly facilitate orthotopic transplantation and subsequent observation, and provide information about general



FIGURE 1. Normal scale in cross section. Darkly stained scale plate underlies cellular elements. Mallory's triple stain.

characteristics of tissue transplantation reactions not easily obtained in other systems.

Mori (1931) was apparently the first to graft goldfish scales by inserting them in empty scale pockets with a fine forceps. This simple technique has been modified in the present study so that reciprocal homografts can be made among many fish at a time, with autograft and regeneration controls. Plucking of a scale from its pocket involves the removal of (1) the epidermis covering the exposed portion of the scale plate; (2) the dermis underlying the epidermis and its contained capillaries, chromatophores, etc.; (3) the osteogenic and fibrogenic cells that invest the scale plate; (4) the scale plate and guanophores (reflecting layer) lying beneath it (FIGURE 1). The greater part of the scale pocket is left intact. If a pocket is left empty, a complete new scale is regenerated at a rate dependent on the water temperature. This is accomplished in less than a month at 28° to 32° C. Throughout this paper the acellular fibrillary plate and bony layer will be called the scale plate, while the whole structure will be termed the scale according to the usage of Goodrich and Nichols.

Tricaine (MS 222 of Sandoz) was found to be a particularly suitable anesthetic for the prolonged operations of multiple scale grafting. Experimental fish placed in the anesthetic at a concentration of 60 mg. per liter of water become quiescent in about 20 minutes. Somewhat more anesthetic is required at water temperatures above 22° C., or after fish have been repeatedly anesthe-



FIGURE 2. Technique of scale grafting showing anesthetized goldfish under microscope.

tized at intervals of a few days. The fish may be kept in this solution for several hours without harmful effect, and such fish recover in a few minutes when returned to normal aquarium water. Operations are performed under a binocular dissecting microscope as shown in FIGURE 2. The petri dish containing anesthetic in 0.8 per cent saline will accommodate two fish at a time lying side by side on a thin sponge. A shallow watch glass with 0.8 per cent saline is used to hold one scale briefly during reciprocal graft exchanges. For convenience, grafts are generally made in the row of scales just above or below the lateral line, using alternate scale positions numbered from the operculum (FIGURE 3). This procedure facilitates the operations as well as subsequent identification of each graft. The cytotoxic products of homografts elicit local inflammation in adjacent recipient tissue, but use of alternate scale positions prevents the grafts from affecting each other directly. Since scales most often do not fit closely into a foreign pocket, a fine scissors is used to trim the



FIGURE 3. Tagged fish showing scale grafts in row above lateral line. Four inflamed homografts on each side of central autograft control.

scale to the proper size prior to insertion. With practice, this can be done precisely and rapidly. If a scale is too small to fit properly into a homologous pocket, a larger scale from the abdomen of the same donor is used instead. The overlapping of adjacent scales holds the grafts in place. Goldfish are remarkably resistant to external infection and no sterile precautions are required during or after the operation. The circuli or growth rings of the scale plate provide an easily observed record of growth and replacement. Differences between ontogenetic and regenerated scales are clear-cut. They have been described and figured by Wunder (1949).

General Characteristics of Transplantation Reactions

In each series of homografts every fish also received an autograft and the donor site was left empty as a regeneration control (FIGURE 3). Each fish is identified with a tag inserted through the caudal peduncle as illustrated. Histological changes in scale grafts were observed *in vivo* under the microscope as well as in stained sections. The reflecting layer of the scale serves as an effective mirror that facilitates detailed observation of the superficial tissues. All 323 autografts made to date have been normally revascularized and have remained so without any inflammation whatever. The time required for restoration of normal circulation in autografts varies from only 1 day at 32° C. to 12 to 15 days at 10° C. At 28° C. and below there is initially slight vasodilation and sluggish circulation in autografts as compared to adjacent normal scales. At lower temperatures this condition may persist for several days be-

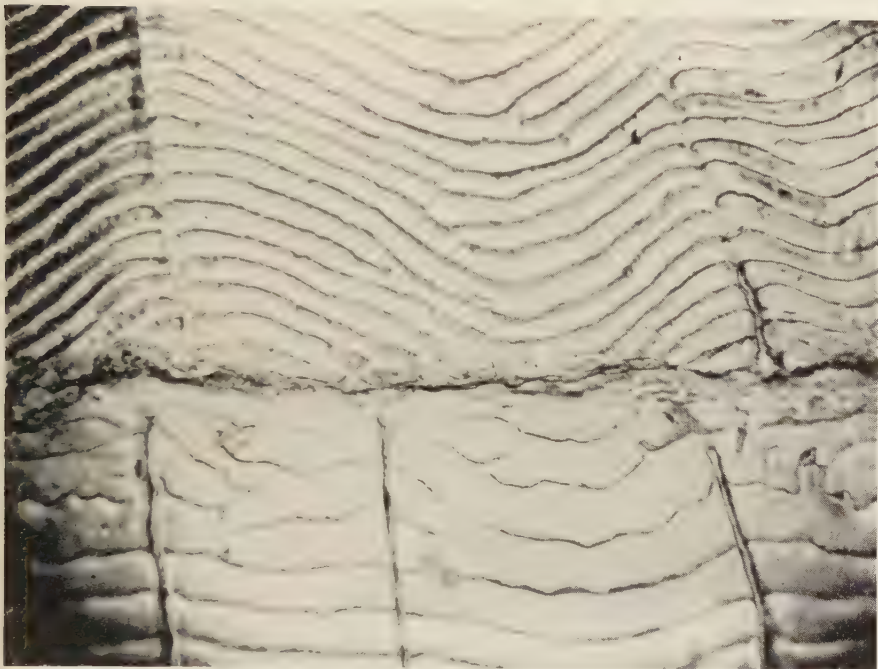


FIGURE 4. Autograft scale plate showing ontogenetic circuli above, and horizontal line along which graft was trimmed with new scale growth below. Alizarin red S stained in 1 per cent aqueous KOH.

fore the circulation becomes completely normal. All autograft tissue elements remain intact. The small area injured by the forceps is repaired, and growth of the scale continues as the fish grows (FIGURE 4).

In contrast, all homografts have been overgrown with hyperplastic host tissue and elicit capillary leakage and vasodilation in the contact zone with recipient tissue. The higher the water temperature, the more rapid and conspicuous are the manifestations of incompatibility. The cellular tissues of the 1978 homografts made thus far have invariably failed to persist. Many first-set homografts are initially revascularized as readily as autografts, but circulation is almost always sluggish, often incomplete, and persists for a few days at most before there is complete stasis. With second-set homografts there is generally no restoration of capillary circulation. In the exceptions observed at 23° C. and 28° C., the circulation lasted for only 24 hours or less. While there is little difference between autografts and homografts for the first few days at 15° C. and below, homografts made at higher temperatures elicit a distinctive hyperplasia within 24 to 48 hours. In experiments done at 28° C. and above, homografts elicit cytotoxic reactions so rapidly that there is conspicuous hemorrhage in the recipient contact zones within 24 hours after grafting. Under the latter conditions circulatory restoration is achieved in only a few first-set homografts and lasts only a day or two. Difference of sex had no

effect on the homograft reaction, nor did differences of age over the interval of 1 to 3 years.

Estimation of the Survival End Point

The experiments now to be described have shown that second-set homografts break down much more rapidly than first-set homografts in all recipient fish. This immune response has been measured by determining the median survival time (MST) and inflammatory reaction of scale homografts under various conditions. Determination of the end point of donor tissue breakdown has been based primarily on biological tests of survival experiments, that is, grafting of scales back to the donor at daily intervals following homotransplantation. Scales transplanted back to the donor after 4 or more days initially evoke inflammation and hyperplasia that persist for several days. This is attributable to the infiltration of the graft by recipient cells that are foreign to the donor. After this crisis any surviving donor tissue is revascularized and persists. It was found in all cases that some donor tissues, including capillaries, survived up to the time that clearing of the dense hyperplastic tissue over the homograft took place, as observed under the microscope. At the time of clearing, only donor chromatophore granules and reflecting tissue remnants in process of sloughing remain on the scale plate. Fortunately the "clearing phenomenon" is quite consistent and can be applied to all phenotypes as a close approximation of the survival end point. In all the time-mortality studies to be described, the survival end point of homografts was judged by the clearing

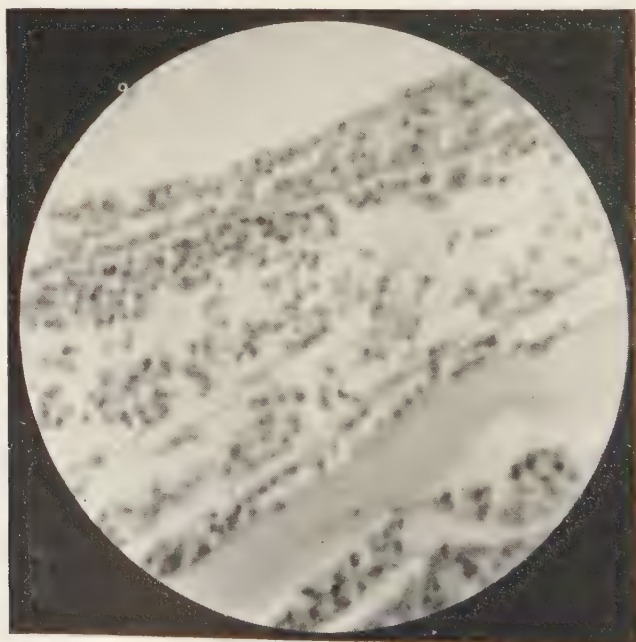


FIGURE 5. Scale homograft at survival end point. Note loss of cytoplasm and lymphocyte infiltration. Hematoxylin-erythrosin B stain.

criterion. The *in vivo* criteria of survival have been corroborated by stained preparations of homografts in progressive stages of breakdown. After Bouin's fixation and infiltration with dioxane and histowax, entire grafts were embedded in histowax and sectioned at $10\ \mu$. Cross sections representing several levels of each graft were stained with Delafield's hematoxylin and lightly counterstained with erythrosin B using the dioxane method. At the time of complete breakdown it is observed that donor cells become pyknotic, then lose cytoplasm, and finally fragment into chromatin droplets (FIGURE 5). The fate of the acellular scale plate will be considered later.

The rapid graphic method of Litchfield (1949) has been used for the time-mortality curves. This method permits use of the data in their original form throughout. Calculations of MST's, standard deviations, and standard sampling errors are performed nomographically after plotting the data on logarithmic-probability paper. The solutions so obtained are as accurate as those derived by the method of probit transformations (Billingham *et al.*, 1954). Time-mortality curves (for example, FIGURE 6) show that the duration and

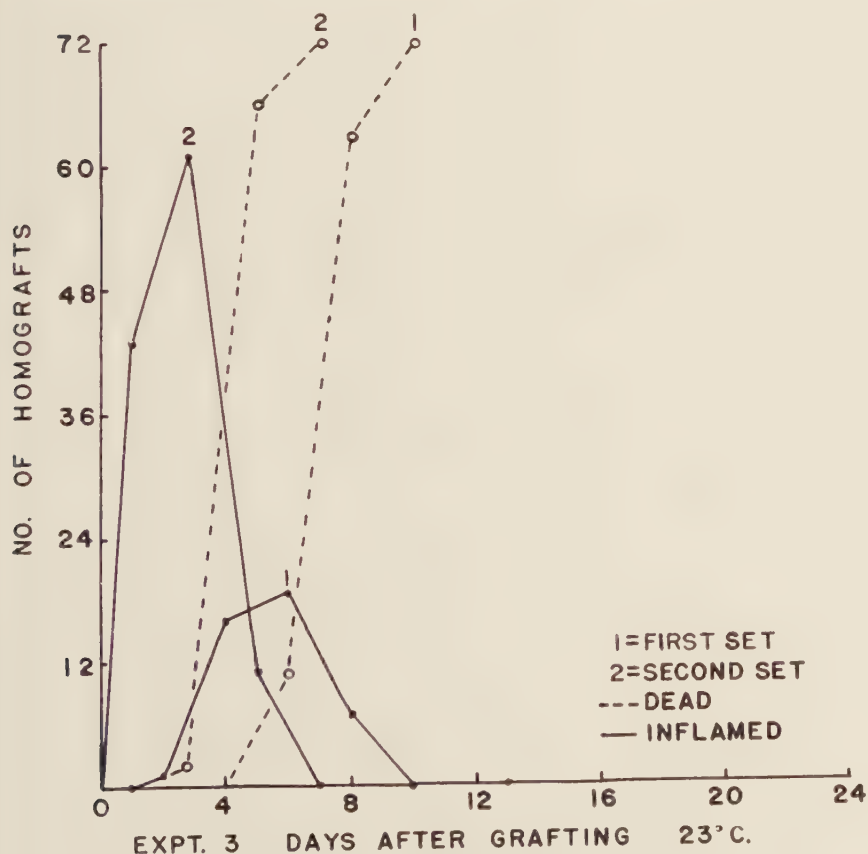


FIGURE 6

intensity of the inflammatory reaction are closely associated with the rapidity of donor tissue destruction. As determined microscopically, capillary hemorrhage in the donor-recipient contact zone always appears earlier and is more severe in second-set homografts. Inflammation is greatly diminished at lower temperatures and capillary hemorrhage did not appear at all in one series of three sets of homografts where tissue breakdown was followed at 8° C. Overgrowth of recipient tissue, however, is associated with homograft destruction at all temperatures. It is more conspicuous at higher temperatures.

Graft Dosage Experiments

Since it was desired to quantitate homograft destruction on the basis of multiple scale grafts to each recipient, any effect of differences in graft dosage on the median survival time had to be determined. Siblings of similar size derived from the same spawning and reared together were used in order to minimize nongenetic differences. Highly inbred lines are not available. Four groups of animals were grafted as shown in TABLE 1, experiments 1 and 2. Each individual in experiment 1 received 8 homografts, while those in experiment 2 each received half this dosage, or 4 homografts. Half of the animals in each experiment were grafted from single donors and the remainder were grafted from 4 different donors. The temperature was maintained at 23° C., and graft destruction was followed by daily microscopic observations. After 26 days, second-set grafts were made, using the same procedure on the opposite side of the body. The results show that in the dosage range tested there is no significant dosage effect on the median survival time of first- or second-set homografts, either when the same donor or different donors are involved. A comparison of time-mortality distributions (last column), that is, of the intervals between survival end points of the first and last grafts in each set to breakdown, reveals a narrow distribution of survival times in first-sets when all

TABLE 1
SCALE HOMOGRAFT SURVIVAL DATA

Expt.	No. of recipient fish	No. of grafts per fish per set	No. of different homo graft donors	Temp. C	Median survival time \pm standard error (days)		Interval between 1st & 2nd sets (days)	Time-mortality distribution (days)	
					1st set	2nd set		1st set	2nd set
1	4	8	1	23	6.6 \pm 0.2	3.5 \pm 0.2	26	4	4
	4	8	4	23	6.8 \pm 0.4	3.9 \pm 0.2	26	9	6
2	5	4	1	23	6.8 \pm 0.2	3.9 \pm 0.2	26	4	4
	5	4	4	23	7.0 \pm 0.3	4.2 \pm 0.2	26	7	3
3	8	9	7	23	6.9 \pm 0.2	3.8 \pm 0.1	25	6	5
4	10	9	9	10	40.5 \pm 1.9	19.5 \pm 0.9	72	41	23
5	10	9	9	15	24.9 \pm 1.2	—	—	25	—
6	10	9	9	16	20.5 \pm 0.9	13.9 \pm 0.5	35	18	13
7	10	9	9	19	12.6 \pm 0.3	8.0 \pm 0.2	30	13	8
8	10	9	9	21	8.3 \pm 0.3	5.4 \pm 0.4	30	11	11
9	9	9	9, 8*	28	6.3 \pm 0.2	4.4 \pm 0.2	30	6	5
10	10	9	9	32	4.3 \pm 0.1	3.2 \pm 0.1	17	4	4

* One fish died after the first-set grafts were made. The graft dosage was kept the same in the second-set by placing two grafts from one donor on each recipient.

grafts are taken from single donors. When diverse donors are used, however, the mortality curve extends over a longer period, which is attributable to the prolonged survival of a few grafts. This difference does not hold in the second-sets since immunity has been developed against all graft tissues. The survival time of individual homografts in the first-set reflects the antigenicity of each graft. Variations of operative technique affecting the healing and vascular penetration of the grafts may be held chiefly responsible for the differences in survival times of grafts transplanted from the same donor to a given recipient.

Effect of Water Temperature on the Median Survival Time

Cushing (1942) demonstrated an effect of temperature upon antibody production in fish. He found that the rate of production of agglutinins against sea urchin sperm in carp and goldfish held at 15° C. and 28° C. was much faster in the fish kept at the higher temperature. Since the growth and metabolism of fish is known to be influenced by the water temperature, it was thought that the survival times of scale homografts might also be temperature-dependent. Several comparable series of grafts were made at various temperatures ranging from 10° C. to 32° C. The fish were of uniform gold phenotype in all experiments except No. 8, where shubunkins were used. Unrelated fish were selected from the large stocks of a local fish farm* to obtain a maximal response to the homografts in each experiment. Fish were tagged when received and acclimated to the desired temperature for 1 week prior to grafting. Reciprocal homografts, along with autograft and regeneration controls, were made among 10 fish at each temperature. Each graft was observed microscopically at intervals of 1 to several days, depending on the rapidity of homograft destruction in each experiment. After the first-set homografts were completely destroyed, a second-set of homografts was made on the opposite side of the body as described earlier. One or 2 homografts out of 90 were often lost from each set within 24 hours of an operation as a result of the activity of the fish before firm healing was achieved. The time-mortality statistics were computed on the basis of the total number of grafts retained. The results are summarized in TABLE 1, experiments 4 through 10. A plot of median survival times against temperature is shown in FIGURE 7. It is evident that the median survival times of both first-set and second-set grafts are closely temperature-dependent. Over the range of 10° C. to 19° C. the first-set MST's decrease linearly with an increase in temperature. Above 19° C., and particularly above 21° C., the rate of homograft destruction increases less rapidly, indicating a modification in the nature of the response to temperature dependence. This is true also of the second-set homografts at higher temperatures. At any given temperature the median survival time of the second-set is approximately one half to two thirds of that observed in the first set. It may be argued that differences in metabolic rate, affecting the rate of digestion of donor tissues by recipient leukocytes, exert the dominant influence in destruction of the second-set as well as the first-set. Even at 32° C., which approaches the maximum temperature at which goldfish may be maintained for prolonged periods, the

* These fish were supplied by the Altadena Water Gardens, Altadena, Calif.

rate of destruction of both sets of homografts is still higher than at 28° C. The rate at which new scales developed in the regeneration control sites also increased with the temperature, as did the rate of revascularization of autografts.

The Second Inflammatory Phase and Scale Plate Digestion

Thus far we have considered the aspects of homograft destruction affecting the cellular or homovital tissues of goldfish scales. In experiments 1, 2, and 3, which involved a single sibship, that is, syngenesiotransplants in the terminology of Loeb (1945), all donor scale plates were eventually reconstituted to normal by the recipients after digestion of the homovital tissues was completed.



FIGURE 7



FIGURE 8. Mosaic donor-recipient scale plate. Partially digested donor plate above and recipient plate below. Alizarin red S stained in 1 per cent aqueous KOH.

As shown in FIGURE 6, there was no renewal of inflammation around either set of homografts after the cellular tissues had broken down. That the donor scale plates did in fact persist was ascertained by periodic examination of the reconstituted scale plates for 7 months after grafting. The proximal concentric circuli observed only on ontogenetic scale plates remained intact on the homografted plates.

In the experiments in which unrelated goldfish were utilized in reciprocal homografting operations, the fate of the scale plate was generally quite different. In a few instances homograft scale plates persisted and were reinvested with soft tissues, as in the sibship described above. In experiment 9, where scale-plate digestion proceeded rapidly at 28° C., it was observed that no homograft plate remained completely intact. About 12 per cent of the re-

constituted scale plates represented a mosaic of donor and recipient elements as judged by the circuli (FIGURE 8) while, in the remaining sites, new scales were regenerated after complete digestion or sloughing of the homograft plates. The course of the inflammatory reaction in this experiment (FIGURE 9) is typical of the response to multiple homografts observed in all the experiments with unrelated fish. At each observation the severity of capillary hemorrhage (redness) in the donor-recipient contact zone was recorded for each homograft as (0), (\pm), (+), or ($++$). Although inflammation is generally more severe in second-set homografts and at the maxima in both sets, the plotted curves account only for the total number of homografts inflamed to any degree (\pm , +, or $++$) at each observation. It should be emphasized that the autograft controls showed no inflammation at any time. One observes that the first inflammation disappears as the cellular elements of the homografts become completely digested. A second inflammatory phase is then initiated as the scale plates come into close contact with outgrowing recipient capillaries. The 2 phases in each set overlap in the cumulative plots because of the different

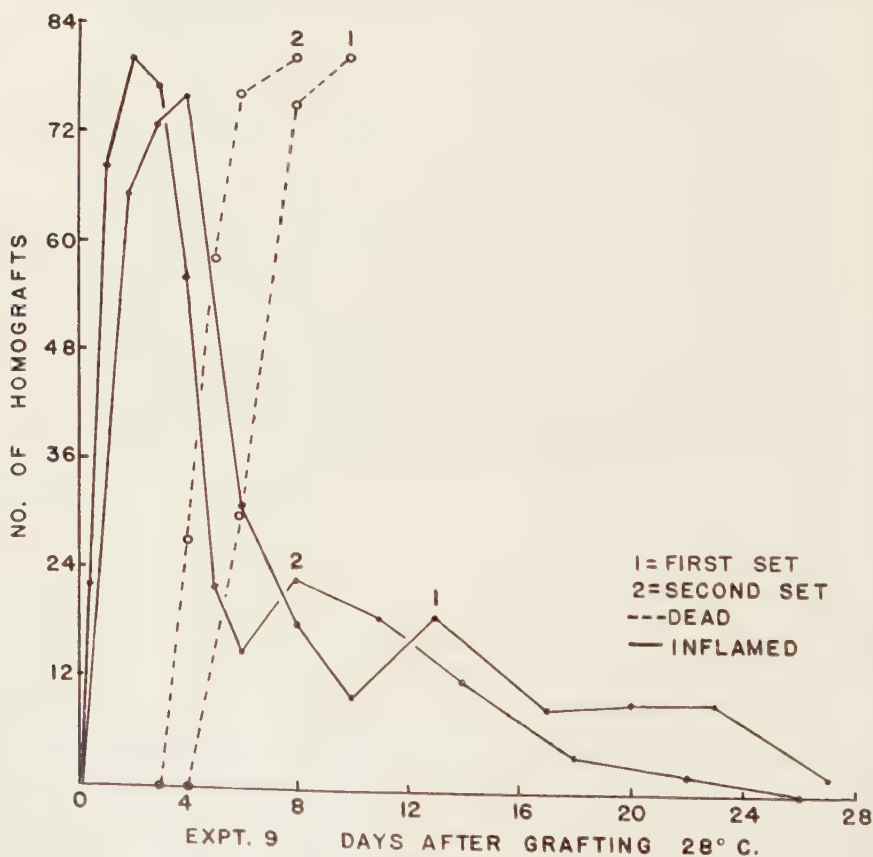


FIGURE 9

rates of homograft destruction. Most of the homograft plates that fail to elicit capillary hemorrhage in the contact zone do cause peripheral hyperplasia and vasodilation during the course of plate digestion. In these instances the recipient is apparently able to remove cytotoxic substances before much local damage is done. A comparison of first-set and second-set inflammatory reactions is nevertheless enlightening. While the second inflammatory phase in the first-set does not reach a peak until the 13th day, the equivalent peak is reached by the second-set on the 8th day. An increased overlap of these phases is also evident in second-set homografts.

Further insight into these phases was obtained by making third-set and fourth-set homografts at 30-day intervals on the animals of experiment 8. The median survival times of the third and fourth sets were 5.2 ± 0.3 days and 5.2 ± 0.4 days, respectively. On this basis there was no significant increase in the rate of the reaction over that observed for the second-set homografts. When the inflammatory curves of the 4 sets are compared, however, it is found that the second inflammatory phase begins earlier and involves more homografts in each succeeding set. The course of this phase in the second and later sets cannot be readily quantitated because the severity of the second phase causes many scale plates to be sloughed at the outset. That the homograft scale plate by itself was capable of inducing an inflammatory reaction was demonstrated by first digesting away the soft tissues of scales in 0.1 N NaOH and then cleaning the resulting scale plates in saline before grafting. Autografts made in this manner at 22° to 24° C. were reinvested with soft tissues, while homografts elicited local hemorrhage, which developed between the 10th and 15th day after grafting, and were eventually sloughed. The time required for inflammation to develop was close to that observed for the second phase with intact homoscales at similar temperatures. All the evidence taken together suggests that at least some of the scleroproteins comprising the scale plate are antigenic, but are only slowly digested by homologous recipients. This digestion always takes place more rapidly in second-set homografts. The lack of such digestion in the syngenesiotransplants studied suggests that the scleroproteins in general may manifest less antigenic diversity than the homovital tissues of the scale. The experiment with treated scales involved only a few fish and it is quite possible that other kinds of treatment in larger experiments would give different results.

The possibility that damaged scale plates might have been autoantigenic or digested when the collagen fibers were exposed directly to the highly vascularized dermis was also investigated. Distal sections of the scales of 4 fish were cut away both *in situ* and in conjunction with autografting. In all cases hyperplastic tissue appeared in the area of the scale pocket where the scale had been cut away, but there was no inflammation. In less than 3 weeks at 23° C., new scleroprotein was formed in all cut-away areas to reconstitute the scales to a normal shape and size.

Discussion

It is rather surprising to find that autografts made at lower temperatures can survive without visible damage for 1 to 2 weeks before capillary circulation is

restored. The thinness of the scale and external contact with cold water apparently facilitate respiration and metabolism in the absence of a dermal blood supply. A similar delay in revascularization is observed in first-set homografts at these temperatures. Here, too, there is no apparent tissue death during the early period of ischemia. The analogous resistance of mammalian skin to anoxia and general ischemia has been considered by Billingham *et al.* (1954). The rate of breakdown of first-set scale homografts was not observed to depend on the length of time during which circulation persisted. The superficial overgrowth of hyperplastic host tissue appears to provide the critical contact with all homografts. This applies especially to second-set homografts, where circulatory restoration is rarely achieved at any time, whereas hyperplastic overgrowth is more conspicuous than in the first-set. From these considerations it is evident that hemal stasis cannot be used as the end point of scale homograft survival, as Taylor and Lehrfeld (1955) have done with rat skin grafts. Any means of estimating survival time must in the last analysis depend on biological tests of survival involving transplantation of homografts back to the original donor.

Although homograft survival time has been shown to be influenced by graft dosage in rabbits (Medawar, 1944) and in rats (Taylor and Lehrfeld, *op. cit.*), the effect obtains only when a single set of regional lymph nodes drain the graft area or when very minute grafts are compared to large grafts. Thus it is not surprising that twofold differences in dosage of scale homografts made along the side of the body have slight, if any, effect on the median survival time. While grafts transplanted to a single recipient from genetically diverse donors survive for very different lengths of time, the time-mortality relationships were found to follow normal, sigmoid curves in cumulative plots. These curves became straight lines when plotted on logarithmic-probability paper. This suggests that the grafts may behave almost independently, since the same number of grafts made singly to separate recipients should also give a log-normal curve. The slopes might differ significantly, however. Recipients from a highly inbred line would have to be used to resolve this question. One might expect that multiple grafts would cooperate in bringing about their destruction to the extent that they share antigens in common. The scale homograft dosage experiments, however, indicate that, at least within the limits studied, the quantity of antigen digested does not modify the MST's of either the first- or second-set grafts. The fact that hyperimmunization with third-set and fourth-set grafts in experiment 8 failed to further shorten the MST found in the second-set is additional significant evidence.

All the cumulative time-inflammation curves show that the duration of the first inflammatory phase is closely associated with the rapidity of donor tissue destruction. When the intensity of hemorrhagic reactions around individual homografts is compared with their survival time, no relationship is evident. Moreover, certain fish were observed to exhibit more severe inflammatory reactions to all homografts than others in the same experiment involving reciprocal exchanges. Yet inflammation is generally more intense around second-set homografts, where the median survival time is much less. It can be stated that inflammation becomes minimal in both sets as the survival end

point for all homografts in an experiment is reached. The second inflammatory phase begins in the grafts first broken down while there is still some surviving tissue in the last grafts to reach the survival end point. The second phase was observed to be a consequence of relatively slow digestion of the scleroproteins comprising the scale plate. Scale-plate digestion also showed the second-set phenomenon.

Summary

(1) Goldfish scales are highly vascularized structures with a complex histology. Their location and mode of attachment greatly facilitate orthotopic transplantation and subsequent observation, and provide information about general characteristics of tissue transplantation reactions not easily obtained in other systems. A dependable technique of scale grafting, with autograft and regeneration controls, has been devised so that reciprocal homografts can be made among many fish at one time.

(2) In all experiments, autografts have been normally revascularized without any inflammation whatever, whereas homografts are rapidly overgrown with hyperplastic host tissue and elicit capillary leakage and vasodilation in the contact zone with recipient tissue. Second-set homografts regularly break down much more rapidly than first-set homografts. This immune response has been measured by determining the median survival time and inflammatory reactions under various conditions.

(3) A plot of median survival times against temperature demonstrated that previous grafting from the same donor and higher water temperature both accelerated homograft destruction. Time-mortality curves show that the duration and intensity of the inflammatory reaction are closely associated with the rapidity of donor-tissue destruction. Capillary hemorrhage in the donor-recipient contact zone always appears earlier and is more severe in second-set homografts.

(4) Cumulative time-inflammation curves demonstrated that the duration of the first inflammatory phase is associated with the rapidity of soft-tissue destruction. The second inflammatory phase was observed to be a consequence of relatively slow digestion of the scleroproteins comprising the scale plate. Both soft-tissue and scale-plate digestion showed the second-set phenomenon.

Acknowledgments

I thank Ray D. Owen for his advice and' generous help throughout these experiments. For several of the better photographic illustrations I am very grateful to Gene Wolfsheimer. The support of a Research Fellowship from the United States Public Health Service, Bethesda, Md., and of Arthur McCallum Summer Scholarships at the California Institute of Technology, Pasadena, Calif., in 1954 and 1955 are also appreciated.

References

- BILLINGHAM, R. E., L. BRENT, P. B. MEDAWAR & E. M. SPARROW. 1954. Quantitative studies of tissue transplantation immunity. I. The survival times of skin homografts exchanged between members of different inbred strains of mice. *Proc. Roy. Soc. London, B.* **143**: 43-58.

- COLE, W. H. 1922. The transplantation of skin in frog tadpoles, with special reference to the adjustment of grafts over eyes, and to local specificity of integument. *J. Exptl. Zool.* **35**: 353-419.
- COLLINS, H. H. & E. F. ADOLPH. 1926. Skin pattern in urodele amphibia. *J. Morphol.* **42**: 473-522.
- CUSHING, J. E. 1942. An effect of temperature upon antibody production in fish. *J. Immunol.* **45**: 123-126.
- GOODRICH, H. B. & R. NICHOLS. 1933. Scale transplantation in the goldfish. *Carassius auratus*. I. Effects of chromatophores. II. Tissue reactions. *Biol. Bull.* **56**: 253-265.
- LITCHFIELD, J. T., JR. 1949. A method for rapid graphic solution of time-per cent effect curves. *J. Pharmacol. Exptl. Therap.* **97**: 399-408.
- LOEB, L. 1945. Biological Basis of Individuality. :1-711. C. C. Thomas, Springfield, Ill.
- MAY, R. M. 1923-24. Skin grafts in the lizard, *Anolis carolinensis*. *Brit. J. Exptl. Biol.* **1**: 539-555.
- MEDAWAR, P. B. 1944. The behavior and fate of skin autografts and skin homografts in rabbits. *J. Anat.* **78**: 176-199.
- MORJ, Y. 1931. On the transformation of ordinary scales into lateral line scales and lateral-line organs in the goldfish. *J. Fac. Sci. Imp. Univ. Tokyo. Sec. IV.* **2**: 185-194.
- NARDI, F. 1935. Das Verhalten der Schuppen erwachsener Fische bei Regenerations- und Transplantationsversuchen. *Arch. Entwicklungsmech.* **133**: 621-663.
- NEAVE, F. 1940. On the histology and regeneration of the teleost scale. *Quart. J. Microscop. Sci.* **81**: 541-568.
- SAUTER, V. 1934. Regeneration und Transplantation bei erwachsenen Fischen. *Arch. Entwicklungsmech.* **132**: 1-41.
- TAYLOR, A. C. & J. W. LEHRFELD. 1955. Definition of survival time of homografts. *Ann. N. Y. Acad. Sci.* **59**(3): 351-360.
- VOGEL, H. H., JR. 1940. Transplantation of autogenous and homologous skin in adult *Rana pipiens*. *J. Exptl. Zool.* **85**: 437-474.
- WUNDER, W. 1949. Beobachtungen über Schuppenregeneration beim Goldfisch (*Carassius auratus*), beim Karpfen (*Cyprinus carpio*) und bei der Brasse (*Abramis brama*). *Arch. Entwicklungsmech.* **143**: 396-407.

Discussion of the Paper

JAMES H. BERRIAN (*Medical Service Corps, United States Navy, Naval Medical Research Institute, Bethesda, Md.*): W. H. Hildemann has taken advantage of a unique experimental material to build up large series of data having quantitative significance. In this way he has brought out some of the more subtle features of reactions to homografts that otherwise would have escaped notice. Moreover, the general behavior of scale transplants undergoing rejection is so reminiscent of the pattern of mammalian skin homograft failure that we can be reasonably confident that the same fundamental reactions are involved in each case.

One of the more obscure phases of the homograft problem concerns the terminal events, in which tissue destruction is brought about by immune mechanisms. I should like to speculate on how sufficient quantitative data of the sort Hildemann has presented on the time course of destruction may provide a description of the mechanisms involved.

The median survival time for scale homotransplants left on the recipients, as determined by the "clearing phenomenon," would seem to coincide closely with the median survival time arrived at by returning grafts to the donors. In the designs used here, either where one of a series of grafts is returned to the donor at daily intervals or in the reciprocal grafting procedures, immunization of the donor against the cells of the recipient investing the graft is brought about. As Hildemann has pointed out, a reaction will ensue against the cells of the recipient when the grafts are returned to the donor. In the biological

test of survival, the end point is based on the persistence of donor cells in the returned mosaic after the reaction against the recipient elements has subsided. It is apparent that any further destruction of donor cells upon return of the graft to the donor will act to give a reduced median survival time. If the median survival time thus determined does not differ significantly from a median survival time estimated for grafts left in place on the recipient, where well-established independent criteria such as the "clearing phenomenon" or the cessation of inflammation have been used as the end point, the coincidence would seem to indicate that further destruction of grafts upon return to the donor does not occur.

Weaver, Algire, and Prehn¹ have found that immune cells from the spleen, presumably bearing antibodies, will lyse antigenic cells when these are enclosed together in diffusion chambers. Lysis will result even when the chambers are placed on isogenous, nonimmune hosts, which are apparently able to provide any required nonspecific cofactors. The importance of nonspecific cofactors was suggested by the absence of lysis *in vitro*. Furthermore, the experiments indicated that lysis resulted from contact of the immune and antigenic cells, with the release of diffusible substances that lysed adjacent cells not in contact. It was not shown whether the diffusible substance was antibody or a nonspecific cytolytic, and either of these might explain the results. It can be pointed out that, even though sufficient antibody might be accumulated within a graft, the availability of the nonspecific cofactors might limit the rate of destruction.

Returning to the comparison of survival times for grafts left on the recipient with those returned to the donors, there exist 2 possibilities whereby the latter would be affected. First, antibodies accumulated within the grafts might continue to bring about lysis, utilizing nonspecific cofactors provided by the donor. Second, if nonspecific lysins are responsible for the destruction, these would be released in the reaction against the recipient cells of the mosaic, and they would affect the donor cells with equal ease. Placing grafts in the process of destruction on previously unimmunized hosts would allow the contribution of the first process mentioned to be evaluated. If the survival times do coincide, however, as Hildemann's results suggest, then it would seem that neither of the possibilities mentioned are operative. It might be concluded that nonspecific cytolytic substances are not involved, but that antibodies released from immune cells invading the graft bring about the destruction directly, with the participation of nonspecific cofactors. Furthermore, it would follow that the rate of destruction would not be dependent on the availability of the nonspecific cofactors, but upon the availability of the antibody.

Reference

- WEAVER, J. M., G. H. ALGIRE & R. T. PREHN. 1955. J. Natl. Cancer Inst. **15** (6): 1737

POSTPARTUM INDUCTION OF TOLERANCE TO HOMOLOGOUS SKIN IN RATS*

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At the previous conference on tissue homotransplantation held here two years ago, Billingham, Brent, and Medawar defined "acquired tolerance" as "an induced state of specific nonreactivity toward a substance that is normally antigenic—a nonreactivity, moreover, that is due to a primary failure of the machinery of the immunological response." They then described how tolerance in respect to homologous tissue, which develops naturally in most dizygotic cattle twins and in a few dizygotic human twins, can be induced in mice, chicks, and rabbits by injecting the recipients, at a sufficiently early stage of their development, with cells from the prospective graft donor.

The period during which tolerance with respect to homologous skin can be induced, although not sharply delimited, as a rule ends a day or two before birth in mice (Billingham, Brent, and Medawar, 1953, 1955), about the time of hatching in chicks (Cannon and Longmire, 1952), and well before the end of intrauterine life in rabbits (Billingham *et al.*, 1955) and sheep (Schinkel and Ferguson, 1953). In rats, however, Woodruff and Simpson (1954, 1955) found that complete, or almost complete, tolerance developed regularly in animals injected on the day of birth and occasionally in animals injected at the age of 2 weeks. It was shown further that administration of cortisone for 12 days, starting on the day of injection of the homologous cells, did not decisively alter the probability that tolerance would be induced, but that when tolerance did occur it tended to be a little more complete in cortisone-treated animals. Of considerable interest was the discovery that, when tolerance was not quite complete, a second graft from the original donor sometimes broke down, while the first graft showed no change. This would seem to add further support to the writer's hypothesis of the critical period (Woodruff, 1952, 1954).

These results have been published elsewhere (Woodruff and Simpson, 1955). For purposes of comparison, however, they have been summarized in TABLES 1 and 2†.

The new material presented here concerns an attempt to retard the development of immunological maturity with respect to the reaction to homologous tissue antigens. It was decided to try administration of cortisone and somatotrophin, because these hormones are known to influence the development of young rats and because their effects are in some respects antagonistic (Parmer, Katonah, and Angrist, 1951). So far, however, only cortisone has been tried.

The general plan was very similar to that employed in the earlier experiments. Six litters of newborn Wistar rats were taken from stock, and the number in each litter was reduced to 8. Each immature rat received a daily injection of 0.05 mg. cortisone, starting on the day of birth and continuing for 14, 21, or 28 days. On the day after the last cortisone injection, each surviving

* This work was aided by grants from the Medical Research Council of New Zealand, Wellington, N. Z.

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TABLE 1
BEHAVIOR OF FIRST-SET SKIN HOMOGRAFTS IN RATS
PREVIOUSLY INJECTED WITH DONOR CELLS

Cortisone Treatment (When Given) was Started on Day of Injection of Donor Cells

Age of recipient when donor cells first injected	Cortisone-treated or not	Number of animals grafted	Number of grafts surviving after:		
			2 weeks	4 weeks	12 weeks
Newborn	No	8	8	8	8
	Yes	11	11	11	11
2 weeks	No	12	3	2	2
	Yes	15	6	4	4
4 weeks	No	8	0	0	0
	Yes	8	0	0	0

animal received a subcutaneous injection of 0.3, 0.45, or 0.6 IU of long-acting ACTH (organon corticotrophin "Z"), according to whether the recipient was 2, 3, or 4 weeks old, and another injection of 0.2 ml. of a cell suspension from an adult hooded rat, prepared from splenic tissue by the technique described previously (Woodruff and Simpson, 1955) and containing approximately 180,000 cells per cu. mm. The dose of cortisone was deliberately made large, and in consequence only about one third of the animals survived. Each of these, when 3 months old, received a split skin homograft (2×2 cm.) from the corresponding hooded donor that has provided the cell suspension. Small portions of the grafts were removed for histological examination after 2, 3, 4, and 12 weeks (see FIGURES 1 to 8).

The results are shown in TABLE 3, from which it will be seen that injection of homologous cells at 2 weeks of age induced a high degree of tolerance. Injection at 3 weeks of age as a rule induced a slight but definite degree of tol-

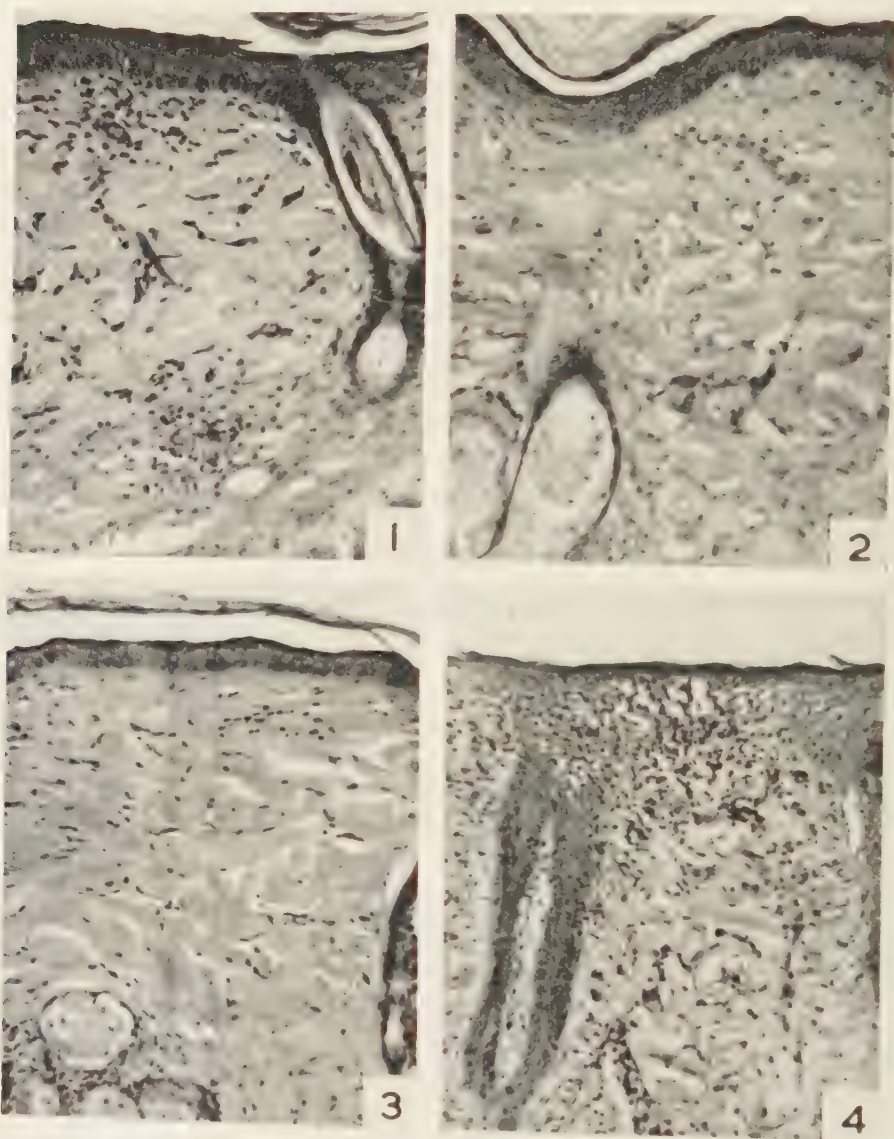
TABLE 2
BEHAVIOR OF SECOND-SET SKIN HOMOGRAFTS IN RATS
PREVIOUSLY INJECTED WITH DONOR CELLS

Cortisone Treatment (When Given) was Started on Day of Injection of Donor Cells

Age of recipient when donor cells injected	Cortisone-treated or not	Survival of first-set graft for 12 weeks or not	Number of animals receiving second-set grafts	Number of second-set grafts surviving after:		
				1 week	2 weeks	4 weeks
Newborn	No	No	0	0	0	0
		Yes	7*	7	6	6
	Yes	No	0	0	0	0
		Yes	10*	10	10	10
2 weeks	No	No	3	2	0	0
		Yes	1*	1	0	0
	Yes	No	5	2	0	0
		Yes	4	4	3	3
4 weeks	No	No	4	0	0	0
		Yes	0	0	0	0
	Yes	No	4	0	0	0
		Yes	0	0	0	0

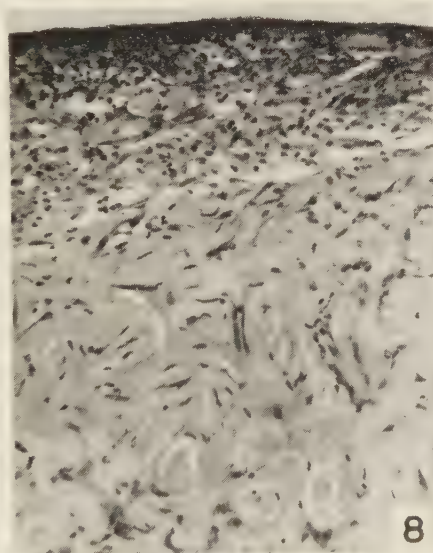
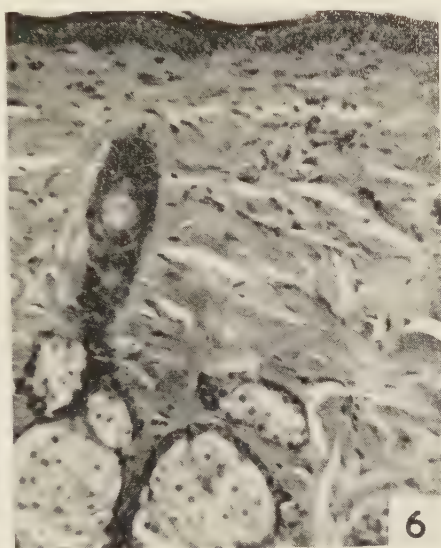
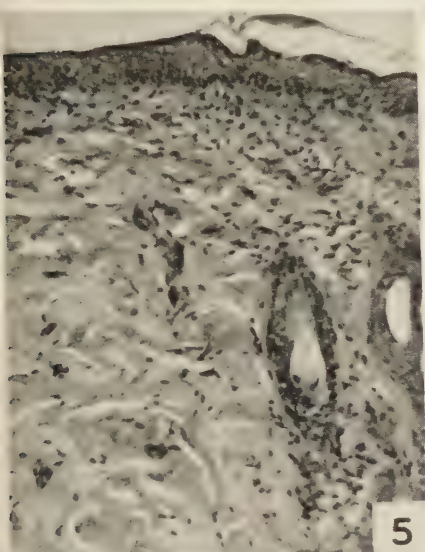
*One animal that failed to survive the operation has been excluded.

erance, but in one animal no tolerance resulted and another became highly tolerant. Animals injected at 4 weeks of age did not become tolerant. It was not determined, however, whether they became abnormally resistant. Some caution is required in interpreting the findings, owing to the rather small



FIGURES 1, 2, 3. Skin homograft after 2, 3, and 4 weeks respectively in a rat made highly tolerant by injection of prospective donor cells at 2 weeks of age following administration of cortisone from birth. H. and E. $\times 175$.
 FIGURE 4. Skin homograft after 2 weeks in a rat in which only a slight degree of tolerance resulted from injection of prospective donor cells at 3 weeks of age following administration of cortisone from birth. Breakdown of the graft is well advanced but not yet complete. H. and E. $\times 175$.

number of surviving animals and the fact that second-set grafts have not yet been studied. It appears, however, that the period during which rats react in an immature way to homologous cells can be extended, slightly but definitely, by administering cortisone from the day of birth.



FIGURES 5, 6, 7. Skin homograft after 2, 3, and 4 weeks respectively in a rat that became highly tolerant as the result of injection of prospective donor cells at 3 weeks of age following administration of cortisone from birth. H. and E. $\times 175$.

FIGURE 8. Skin homograft after 2 weeks showing complete breakdown. The host was injected with prospective donor cells at the age of 4 weeks following administration of cortisone from birth. No tolerance. H. and E. $\times 175$.

TABLE 3

BEHAVIOR OF FIRST-SET SKIN HOMOGRAFTS IN RATS
PREVIOUSLY INJECTED WITH DONOR CELLS

Cortisone Given from Birth up to Day of Injection of Donor Cells

Age of host when donor cells injected	Number of animals	Number of grafts surviving after:			
		2 weeks	3 weeks	4 weeks	12 weeks
2 weeks	4	4	4	4	
3 weeks	6	5	2	1	
4 weeks	7	0	0	0	

There are at least 3 ways in which these investigations might profitably be extended.

In the first place, it is desirable to know whether tolerance can be induced in the human infant at birth. A priori, this would seem to be unlikely because the human infant is relatively mature at birth, but speculation is no substitute for experiment. In my unit we plan to make skin grafts, taking the skin from the corresponding blood donor, to children who received blood transfusions at or soon after birth for hemolytic disease of the newborn.

Second, animals that are rendered highly tolerant provide valuable material for a full-scale investigation of the critical-period hypothesis. Billingham *et al.* found that tolerance could be abolished, and a well-established graft destroyed after it had been *in situ* for "at least 75 days," by transplantation of lymphoid-tissue from an animal isogenic with the host, especially if this animal had been immunized against the cells of the graft donor. It is pertinent to ask, however, whether the phase during which this phenomenon can be demonstrated persists throughout the life of the host. The persistence of three first-set grafts in highly but not completely tolerant rats, despite the breakdown of second-set grafts in the same animals, provides ample justification for reopening the question and undertaking further experiments.

Finally, the fact that tolerance can be induced for a short time after birth in rats, and the demonstration that administration of cortisone delays to some extent the development of immunological maturity, should encourage further investigations of this kind. If some more effective way of retarding development in this particular respect could be found, it should be possible, by determining what other developmental processes are affected, to deepen our understanding of the mechanism by which homografts are normally destroyed.

References

- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature*. **172**: 603.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1955. Acquired tolerance of skin homografts. *Ann. N. Y. Acad. Sci.* **59**(3): 409.
- CANNON, J. A. & W. P. LONGMIRE. 1952. Studies of successful skin homografts in the chicken. *Ann. Surg.* **135**: 60.
- PARMER, L. G., F. KADONAH & A. A. ANGRIST. 1951. Comparative effects of ACTH, cortisone, corticosterone, desoxycorticosterone, pregnenolone on growth and development of infant rats. *Proc. Soc. Exptl. Biol. Med.* **77**: 215.
- SCHINKEL, P. G. & K. A. FERGUSON. 1953. Skin transplantation in the foetal lamb. *Australian J. Biol. Sci.* **6**: 533.

- WOODRUFF, M. F. A. 1952. The transplantation of homologous tissue and its surgical applications. *Ann. Roy. Coll. Surg. Engl.* **11**: 173.
- WOODRUFF, M. F. A. 1954. The "critical period" of homografts. *Trans. Bull.* **1**: 221.
- WOODRUFF, M. F. A. & L. O. SIMPSON. 1954. Induction of acquired resistance to homologous tissue. *Proc. Univ. Otago Med. School.* **32**: 12.
- WOODRUFF, M. F. A. & L. O. SIMPSON. 1955. Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor shortly after birth. *Brit. J. Exptl. Pathol.* **36**: 494.

Discussion of the Paper

RUPERT BILLINGHAM (*University College, London, England*): Woodruff is to be congratulated for giving us a method whereby a high degree of tolerance with respect to skin homografts may consistently be induced in one common laboratory mammal. The great advantage of the method lies in the fact that it does not entail the inoculation of embryos *in utero*, and that it therefore completely eliminates the high mortality normally attending this procedure.

Woodruff's choice of experimental subject appears to have been a particularly fortunate one because of the unexpectedly high degree of immunological immaturity of the neonatal rat, which differs profoundly in this respect from the newborn mouse, or indeed from all other newborn mammals and birds that have so far been studied.

Tolerance of tissue homografts is the outcome of exposure of animals to living tissue-cell antigens in the period of development before the functional differentiation of their immunological response mechanism occurs. The upper limit of the tolerance range—the period in an animal's life during which inoculation with foreign cells may induce tolerance (as opposed to immunity)—approximates the time of birth in the mouse. Tolerance results in fewer than 10 per cent of mice inoculated within a few hours of birth. In the majority of mice, birth represents a "neutral period," during which inoculation induces neither tolerance nor immunity with respect to skin homografts transplanted in adult life. In chickens, too, as the work of Cannon and Longmire shows, the upper limit of the tolerance range barely exceeds the time of hatching. In rabbits, sheep, and cattle there is evidence that the tolerance range (so far as homologous cellular antigens are concerned) terminates at relatively much earlier stages of embryonic life, so that these newborn animals give the adult modality of response.

In view of the fact that from the time of birth animals must assume responsibility for their own immunological defense, it is most surprising that the "neutral period" in the rat, as Woodruff's results show, is not attained until about the 14th day postpartum. Halliday's finding, that in this species, transfer of maternal antibodies by way of the milk continues actively throughout lactation until it terminates rather abruptly at 21 days, may have some relevance here.

Because of the great anatomical immaturity of their young at birth, marsupials have suggested themselves as convenient subjects for the induction of tolerance. It now appears, however, that the postulated advantage of such inconvenient and notoriously difficult laboratory animals as opossums is possessed by rats, perhaps the most commonplace of all laboratory animals.

Despite the ease with which tolerant rats may be obtained, their use for

experimental purposes is restricted because at least some reputedly isogenic strains are unreliable. Tolerance is strongly donor-specific; a tolerant rat is unlikely to accept tissues from any rat other than the donor of its neonatal inoculum. There appears to be one possible way in which the tolerance spectrum might be broadened: if, instead of inoculating newborn animals with spleen cells from a single donor, they are inoculated with cells from the pooled spleens of many donors, tolerance might be induced with respect to most of the tissue antigens represented in a closed colony of any given donor strain.

It should be mentioned that the principle of administering cortisone to prolong the tolerance range—presumably by delaying the maturation of the immunological response mechanism—was introduced and successfully employed by Cannon and Longmire. These investigators found that the administration of this hormone to newly hatched chicks increased the proportion of skin homografts transplanted to them, which gave evidence of prolonged survival.

In conclusion, I should like to suggest a possible explanation for Woodruff's observation that a second skin homograft transplanted to an apparently tolerant animal sometimes broke down, whereas a first graft from the same donor continued to survive. It may be added that similar findings had previously been reported by Weber, Cannon, and Longmire in the case of skin homografts in chickens. The vulnerability of homografts to low-grade reactions, such as may eventually be elicited when tolerance is incomplete, is probably heightened during the early stages of healing-in, when a weak immunological opposition may be reinforced by a nonspecific inflammatory reaction of traumatic origin. In such incompletely tolerant animals, during the primary healing phase, second-set homografts are likely to experience a greater disadvantage than well-established first-set grafts. Occasionally, therefore, one might expect to find that the incompleteness of tolerance (that is, the weakness of immunological opposition) was such that an established first-set graft would just be able to withstand it, whereas a newly transplanted graft might succumb because of its handicap, with the additional hazard of traumatic inflammation. Another advantage favoring the survival of the first graft is the fact that its dermis and vascular endothelium will have, to some extent, become repopulated by cells of host origin. Since, as Taylor and Lehrfeld have shown, the initial reaction of a host against a graft manifests itself in the walls of the graft vessels, their repopulation by host cells should to some extent mitigate the effect of the reaction.

We have been completely unable to confirm this finding. In our experience with both mice and chicks, animals that were completely tolerant of a first-set graft invariably fully accepted a second-set graft.

STUDIES ON EPIDERMAL CELL SUSPENSIONS, WITH PARTICULAR REFERENCE TO PROBLEMS OF TRANSPLANTATION IMMUNITY

By R. E. Billingham*

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By the tryptic digestion of thin shavings of skin of even thickness, the dermis can be completely disengaged from the superficial epidermis, leaving the latter as an intact, though rather delicate, sheet of tissue (P. B. Medawar, 1941). Treatment of pure epidermis with an 0.8 per cent solution of sodium citrate in normal saline for a few minutes weakens the cohesion of the Malpighian cells so that they may be detached by light scraping and taken up as a coarse suspension in citrate saline. Vigorous pipetting will completely dissociate any cell aggregates still present. Dissociation in this manner does not destroy the vitality of the cells for, when "grafted" to an appropriate type of full-thickness cutaneous recipient area, they proliferate and regenerate normal epidermis (R. E. Billingham and J. Reynolds, 1952).

This paper is concerned with the use of dissociated Malpighian cells in studies on 2 problems of transplantation immunity carried out in collaboration with Elizabeth Sparrow (R. E. Billingham and E. M. Sparrow, 1954; 1955).

Subjects of the experiments were adult male rabbits, deliberately chosen for their heterogeneity so that the genetic disparity between donors and recipients should be maximal—the theoretical requirement for uniformity of response when inbred strains are not available.

An Attempt to Demonstrate the Formation of "Protective" Isoantibodies in Response to Skin Homografts

As an essential preliminary, the antigenic effectiveness of pure epidermal "grafts" was established. It was found that homografts of epidermis, transplanted orthotopically, elicited and succumbed to an immunity reaction closely resembling that provoked by normal skin homografts. The median survival time of the epidermal grafts was comparable with that of split-thickness skin grafts of comparable aggregate area.

The method employed to look for cytotoxic activity in putatively immune sera was simple. Not less than 2 ml. of the freshly prepared immune serum from a specifically immunized animal was added to a suspension of about 5 to 6 million of a donor's Malpighian cells in 1 ml. of citrate saline. The animal was immunized by making it the recipient, on 2 consecutive occasions about 15 days apart, of a group of 9 fitted "pinch" grafts of its donor's skin. The serological test was performed immediately after regression of the second set of grafts.

Each test was controlled by mixing a similar suspension of the donor's cells with fresh serum prepared from an unimmunized rabbit. After the cells had been exposed to the sera for periods ranging from 1 to 45 hours, during which time they were periodically redispersed in the medium by shaking, they were

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TABLE 1
VITALITY TESTS ON EPIDERMAL CELL SUSPENSIONS AFTER EXPOSURE
in Vitro TO SERUM FROM SPECIFICALLY IMMUNIZED ANIMALS

Test	Duration of treatment (hours)	Temperature of treatment (degrees C.)	Amount* of epithelium produced on grafting from:	
			Experimentally treated cells	Controls
1	1	37	+++	+++
2	2	37	+++	+++
3	3	37	+++	+++
4	2	37	+++	+++
5	2½	37	+++	+++
6	3½	37	+++	+++
7	24	37	+	++
8	24	37	0	+++
9	27½	37	+++	+++
10	29	37	+++	+++
11	23	37	0	+++
12	29	20	+	+++
13	22	5	0	+++
14	27½	5	0	++
15	30	5	++	+++
16	28	5	++	++
17	29	5	+	+++
18	29	5	0	++
19	45	5	+	+++
20	29	5	0	++
21	26	5	+	+++

* Method of scoring epithelium produced by the grafted epidermal cells:

0 = No trace of epithelium after 15 days.

++ = Very small amount of epithelium—as from single minute focus of outgrowth.

+++ = Intermediate amount of epithelium—less than that normally obtained from untreated cells.

++++ = Extensive patch or numerous foci of epithelium—such as obtained from untreated epidermal cell suspensions.

recovered by centrifugation and resuspended in the minimal amount of the supernatant. The effect of the exposure to immune serum on the viability of the cells was then determined by distributing them and their controls evenly over the centers of 2 vascular beds each about 6×4 cm.—prepared in the skin of the side of the original donor's chest. Inspections were carried out at regularly spaced intervals between the 9th and 15th days. The number of individual foci of epithelial outgrowth that appeared and the extent of the patch of epithelium to which they had given rise by coalescence by the 13th day were made the basis of the method of scoring adopted.

The results of the 21 independent tests are set out in TABLE 1.

Treatment of the cells *in vitro* for a few hours at 37° C. was without perceptible effect. After prolonged exposure at either 5° C. or 37° C., however, the outcome of no less than 12 of 15 tests indicated that the vitality of the cells exposed to immune sera was inferior to their controls (FIGURE 1). The results of 6 of these tests were absolutely decisive, since the treated cells failed to produce any epithelium. In no test was the result of a control inferior to its experiment. It may be somewhat significant that, in most of the successful tests in which some epithelium was produced by the treated cells, it appeared slightly later than in the case of the control. There were no grounds for sup-

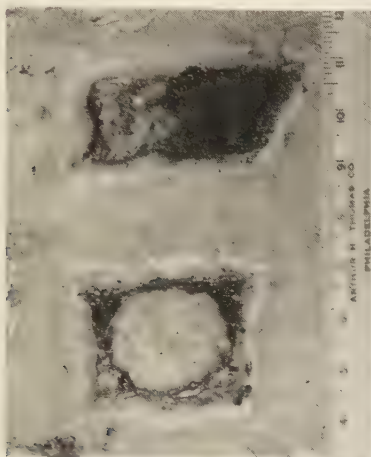


FIGURE 1. Test No. 17. Illustrating the grafting test of viability of a donor's dissociated Malpighian cells after treatment *in vitro* with immune serum for 24 hours at 5° C. The upper recipient area was grafted 13 days previously with the experimentally treated cells, and the lower one with their controls. Only a minute focus of epithelial outgrowth has appeared on the upper site, whereas an extensive sheet of epidermis covers the center of the lower site.

posing that treatment at 37° C. was more effective than at 5° C. There is a slight suggestion, however, that the latter temperature was superior.

On the basis of these findings the formation of "protective" isoantibodies in response to skin homografts is inferred. As microscopy revealed no cytological damage in any of the treated cells, there was no evidence that the antibodies were cytotoxins. Indeed, the existence of a cytotoxic isoantibody, demonstrable *in vitro*, has yet to be revealed.

Essentially similar results were obtained by Gorer (1942) with homologous leukemic cells in mice and, more recently, by Mitchison and Dube (1955) and Mitchison (1955a), in the case of a murine sarcoma and a transplantable lymphoma, 6 C3HED. The weakness of the protective effect of these isoimmune sera (*cf.* the prolonged periods of exposure required in the present experiments) is such, as Mitchison (1955a) has emphasized, that it could easily be missed. Todd and Kidd's (1954) careful tests had previously failed to demonstrate protective antibody formation against tumor 6 C3HED. That this type of antibody plays no more than a minor role in the breakdown of homografts *in vivo* is suggested by the failure of attempts to transfer transplantation immunity passively by means of serum even when repeated massive dosages were employed (N. A. Mitchison 1954, 1955a; R. E. Billingham *et al.*, 1954). It is likewise suggested by the continued survival of susceptible cells in diffusion chambers inserted intraperitoneally in specifically immunized mice (G. H. Algire *et al.*, 1954; J. M. Weaver *et al.*, 1955). Indeed, there is evidence that the administration of immune serum may actually prolong the life of homografts (N. Kaliss and N. Molomut, 1952; N. A. Mitchison, 1955b).

A possible explanation of our results is that the application of immune serum antibody to the Malpighian cells *in vitro* caused them to provoke and succumb to nonspecific vascular-inflammatory reactions on subsequent transplantation (P. B. Medawar, 1948; 1954).

The Influence of Prior Intravenous Injections of Epidermal Cells on the Fate of Skin Homografts

In 1946b Medawar reported that in rabbits the immunizing effect of homologous leukocytes to subsequent skin homografts is at least 18 times more effective through the intradermal route than through the intravenous. In the present study it was the intention of myself and of those associated with me in this work to see whether this finding also applied when homologous Malpighian cells were employed as living cellular antigens.

Control data. Skin homografts in the dosages employed do not normally survive for longer than 9 days (R. E. Billingham *et al.*, 1951), and survival times exceeding 12 days may be taken as strongly indicative of an experimentally modified reaction. A state of immunity in a host is identified by the accelerated breakdown of skin homografts from its donor—they rarely survive longer than 6 days—and by their complete mitotic indolence (P. B. Medawar, 1944; 1946a).

In no animal did the prior injection, given through the marginal ear vein, with dosages ranging from about 1 to 15 million of its intended donor's Malpighian cells, suspended in about 5 ml. of citrate saline, prejudice the survival of a standard set of 5 pinch grafts—each about 9 mm. in diameter—of that donor's skin transplanted in open style 22 days later (FIGURE 2). The reinforcement of the initial antigenic stimulus on the 12th day by a second stimulus of comparable magnitude was without the slightest effect in this respect. Indeed, in a proportion of animals the pretreatment had precisely the *opposite* of the anticipated effect, inasmuch as the survival time of the grafts was significantly prolonged.

Unfortunately, in our early experiments the injections were accompanied by a high proportion of sudden deaths. No fatalities resulted, however, if

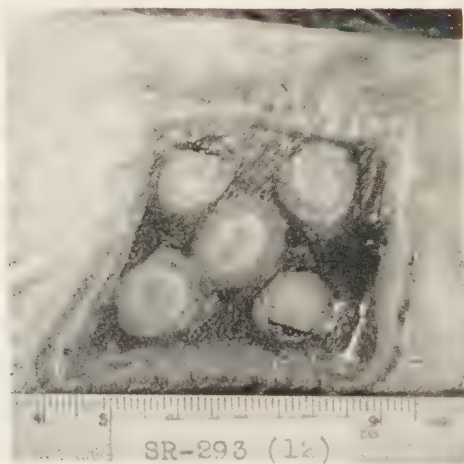


FIGURE 2. A group of 5 skin homografts 12 days after transplantation in "open style" to an extensive recipient area prepared in the skin of the side of the chest on animal SR-293, which had received prior intravenous injections of its intended donor's epidermal cells. The grafts, now ringed by broad annuli of epithelial outgrowth are in perfect condition.

TABLE 2

SURVIVAL TIMES OF SKIN HOMOGRAFTS TRANSPLANTED TO ANIMALS PREVIOUSLY INJECTED WITH THEIR DONOR'S EPIDERMAL CELLS

Animal No.	No. of cells (millions) injected at:		Survival time of skin homografts (days)	
	Primary injection	Secondary injection	First set	Second set
SR-261	10-12*	12-15*	20	7
SR-272	10-12*	12-15*	16	8
SR-275	12-15*	12-15*	16	8
SR-284	10.0	10.0	19	12
SR-293	11.3	10.4	19	13
SR-306	10.5	11.0	22	17
SR-310	13.7	11.3	22	10
SR-314	11.0	11.3	8	—
SR-320	10.4	11.5	20	6
SR-324	10.5	11.3	20	8

* Number of cells estimated from area of epidermis used to prepare suspension. In the remaining experiments numbers of cells are based on hemocytometer counts.

instead of injecting freshly prepared cell suspensions "crude suspension"—in citrate saline, the cells were washed before injection. Something liberated from the epidermis in the course of its dissociation was obviously implicated.

The following procedure was the outcome of numerous empirical attempts to define a regimen of pretreatment that would consistently prolong the life of skin homografts:

Each animal received a primary injection of 10 to 15 million washed homologous epidermal cells, followed by a second injection of a similar quantity of cells 12 days later. This second injection differed from the first in that it comprised a proportion—about one fourth by volume—of the "crude" cell suspension (to include a higher proportion of the "crude" suspension would have been dangerous for reasons already stated). Animals were tested with skin homografts 22 days after the primary injection.

Such was the uniformity of response that the survival of the grafts in 9 of 10 animals was significantly prolonged—in the majority, by a factor of 2 or more (TABLE 2).

The importance of including some of the crude suspension in the second injection was made clear by our failure to obtain prolongation of graft survival in 3 animals that had received the standard pretreatment, but only washed cells were administered on the second occasion. It may be added that even repeated washing did not impair the vitality of the cells. The inclusion of "washings" in the inocula, though desirable, was not obligatory for, in preliminary experiments, prolongations of graft survival were sometimes obtained following the inoculation of animals with washed cells without fortification with crude suspension.

The weakened response of a host as a consequence of its pretreatment was found to be persistent. When animals were again challenged with their donors' skin, a few weeks after the regression of grafts whose survival had been prolonged, none behaved as typical immune animals. In some instances the survival times of these second-set grafts were such that they would have been

considered as significant prolongations had they been first-set grafts (TABLE 2). In most animals, prolongation appeared to be merely the outcome of a delay in the time of onset of an otherwise perfectly normal homograft reaction. In others, however, the actual histological pattern of the reaction was modified, so that it seemed to be directed primarily against the graft dermis, for outgrowing epithelium from the grafts outlived the graft centers by several days.

The results of a preliminary analysis of the phenomenon may be summarized briefly as follows:

(1) Prolongation is not merely the nonspecific outcome of pretreatment of a host with epidermal cells as such, for pretreatment of a rabbit with suspensions of its *own* epidermal cells was without influence on the survival of skin homografts.

(2) Vitality on the part of the epidermal cells at the time of injection appears to be obligatory, for no weakening of an animal's response was demonstrable after its injection with inocula normal in all respects save that, before administration, they had been frozen and thawed under conditions known to be lethal to dissociated cells (R. E. Billingham and P. B. Medawar, 1952).

(3) The intravenous route was obligatory, for the standard inocula administered intradermally elicited a typical immune response, and intraperitoneal inoculation was without influence on the fate of skin homografts.

(4) The effect is not tissue-specific in that pretreatment with the cellular ingredients of skin is not essential to weaken a host's response against skin homografts. Good prolongations of survival have been obtained in hosts pretreated with small amounts of their donor's citrated blood in which the number of leukocytes present was comparable with the number of epidermal cells known to be effective. Unfortunately, despite a considerable amount of work, we have been unable to define a regimen of pretreatment with blood that will give consistent results. Even very high dosages of blood—containing up to 700 million leukocytes [red cells do not elicit transplantation immunity (P. B. Medawar, 1946b; R. E. Billingham *et al.*, 1956a)] administered intravenously in divided doses over a period of 10 days, did not curtail the survival of skin homografts.

Discussion

None of our experimental results offers a clue to the mechanism of the process whereby a rabbit's resistance to tissue homotransplants may be profoundly weakened as a result of its prior intravenous inoculation with a relatively small quantity of living cells from its intended skin donor. The phenomenon is obviously a candidate for inclusion in that category of specifically abrogated immunological responses that includes "enhancement" of the growth of homologous tumors in normally resistant rabbits, rats, and mice as a consequence of their pretreatment with homogenates, desiccates, or extractives prepared either from the tumor concerned or from normal tissue of similar genetic constitution (S. Flexner & J. W. Jobling, 1907; A. E. Casey, 1941; N. Kaliss, 1955; G. D. Snell, 1954). Our finding (R. E. Billingham *et al.*, 1956b) that prior intraperitoneal inoculation of rabbits with lyophilized homologous kidney

may result in an "enhancement" of the survival of skin homografts similar in magnitude to that obtained by the living-cell therapy described in this contribution, lends support to this view. It must be emphasized, however, that "enhancement" differs from the phenomenon reported in this paper in so far as it has been obtained by the administration of *nonliving* material—usually in comparatively large amounts by the intraperitoneal route.

No particular significance should be read into the fact that, whereas "enhancement" of an otherwise resistant host frequently allows a tumor to grow progressively, intravenous injection of a rabbit with living cells has only increased the survival of skin homografts by a factor of about 2. It is generally accepted that, once established, many tumors can override a degree of immunological opposition sufficient to bring about the breakdown of a skin graft. Evidence of this finding has been presented by Billingham, Brent, and Medawar (1956a).

References

- ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1954. Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in immunized mice. *J. Natl. Cancer Inst.* **15**: 493.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc. Roy. Soc. B.* **143**: 58.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1956a. Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Phil. Trans. B.* **239**: 357.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1956b. "Enhancement" in normal homografts, with a note on its possible mechanism. *Transplant. Bull.* **3**: 84.
- BILLINGHAM, R. E., P. L. KROHN & P. B. MEDAWAR. 1951. Effect of locally applied cortisone acetate on survival of skin homografts in rabbits. *Brit. Med. J.* **2**: 1049.
- BILLINGHAM, R. E., & P. B. MEDAWAR. 1952. The freezing, drying and storage of mammalian skin. *J. Exptl. Biol.* **29**: 454.
- BILLINGHAM, R. E., & J. REYNOLDS. 1952. Transplantation studies on pure epidermal epithelium and on epidermal cell suspensions. *Brit. J. Plastic Surg.* **5**: 25.
- BILLINGHAM, R. E., & E. M. SPARROW. 1954. Studies on the nature of immunity to homologous grafted skin, with special reference to the use of pure epidermal grafts. *J. Exptl. Biol.* **31**: 16.
- BILLINGHAM, R. E., & E. M. SPARROW. 1955. The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homografts in rabbits. *J. Embryol. Exptl. Morphol.* **3**: 265.
- CASEY, A. E. 1941. Experiments with a material from the Brown-Pearce tumor. *Cancer Research* **1**: 134.
- FLEXNER, S. & J. W. JOBLING. 1907. On the promoting influence of heated tumor emulsions in tumor growth. *Proc. Soc. Exptl. Biol.* **4**: 156.
- GORER, P. A. 1942. The role of antibodies in immunity to transplanted leukaemia in mice. *J. Pathol. Bacteriol.* **54**: 51.
- KALISS, N. 1955. Induced alteration of the normal host-graft relationships in homotransplantation of mouse tumors. *Ann. N. Y. Acad. Sci.* **59**(3): 385.
- KALISS, N. & N. MOLOMUT. 1952. The effect of prior injections of tissue antisera on the survival of cancer homografts in mice. *Cancer Research.* **12**: 110.
- MEDAWAR, P. B. 1941. Sheets of pure epidermal epithelium from human skin. *Nature.* **148**: 783.
- MEDAWAR, P. B. 1944. Behaviour and fate of skin autografts and homografts in rabbits. *J. Anat.* **78**: 176.
- MEDAWAR, P. B. 1946a. Immunity to homologous grafted skin. I. The suppression of cell division in grafts transplanted to immunized animals. *Brit. J. Exptl. Pathol.* **27**: 9.
- MEDAWAR, P. B. 1946b. Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. *Brit. J. Exptl. Pathol.* **27**: 15.

- MEDAWAR, P. B. 1948. Tests by tissue culture methods on the nature of immunity to transplanted skin. *Quart. J. Microscop. Sci.* **89**: 239.
- MEDAWAR, P. B. 1954. General problems of immunity. *In* Preservation and Transplantation of Normal Tissues. : 1. Ciba Foundation Symposium. Churchill. London, England.
- MITCHISON, N. A. 1954. Passive transfer of transplantation immunity. *Proc. Roy. Soc. B.* **142**: 72.
- MITCHISON, N. A. 1955a. Iso-antibody against a tumor. *Transplant. Bull.* **2**: 93.
- MITCHISON, N. A. 1955b. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J. Exptl. Med.* **102**: 157.
- MITCHISON, N. A. & O. L. DUBE. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. II. The reaction between hemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations. *J. Exptl. Med.* **102**: 179.
- SNELL, G. D. 1954. The enhancing effect (or actively acquired tolerance) and the histocompatibility-2 locus in the mouse. *J. Natl. Cancer Inst.* **15**: 665.
- TODD, J. E. & J. G. KIDD. 1954. Tests for isoantibodies in conjunction with guinea-pig serum against 6C3HED lymphoma cells. *Proc. Soc. Exptl. Biol.* **86**: 865.
- WEAVER, J. M., G. H. ALGIRE & R. T. PREHN. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. *J. Natl. Cancer Inst.* **15**: 1737.

Discussion of the Paper

MORRIS K. BARRETT (*National Cancer Institute, Bethesda, Md.*): In discussing Billingham's paper, I want first to congratulate him on its excellence. Second, I should like to raise a question. What weight does he assign to the possibility that the long incubation *in vitro* was a major nonspecific influence, and that it might enter into the interpretation of the results? Third, I should like to introduce a skeptical attitude by discussing 3 points that have been mentioned. This skeptical attitude is not an acute response to what Billingham has written. Rather, it is a chronic attitude generated by the state of knowledge in this very controversial field. One is often confronted by 2 sets of data, each of which seems credible, yet which, when taken together, appear in conflict. Perhaps we should recall the story of the blind men who encountered an elephant for the time. You remember that the one who examined the leg said that an elephant was like a tree, whereas the one who examined the trunk reported that it was more like a snake.

Such antinomies are common in this field, and the work just reported touches upon 3 special controversies. I mention them not to augment confusion, but rather to diminish the effects of a confusion that already exists. One way to minimize the harm that confusion creates is to recognize its existence.

In this work we have seen again what appears to be the action of a cytotoxin. Much could be said about this, but I shall limit myself to remarking that this demonstration involved an *in vitro* stage, as have all, or nearly all, experiments that gave a positive result. The combined *in vitro-in vivo* technique is a useful one, but in interpreting results one should recall some contradictory evidence. In tissue culture, cells can survive in the presence of tissue or serum from an immune animal that would destroy the same cells *in vivo*. The older literature contains many examples of failure to find cytotoxins in the sera of immune animals. A case in point can be found in the work of Weaver, Algire, and Prehn,¹ who showed that when target cells were combined with normal splenic

tissue and placed in porous chambers, which in turn were put into the abdomens of immunized animals, the target cells survived. Taken together, these data suggest that the presence of a cytotoxin in the host may be no proof that the rejection of a graft depends upon such an antibody. I do not wish to belabor this because my purpose is neither to prove nor to disprove the point.

I merely mention, in passing, the point regarding enhancement following intravenous injections, whereas resistance has followed injection by other routes. There are contradictory reports on this phenomenon.

Regarding the statement that cells must be viable at the time of injection in order to produce enhancement, I have evidence from my own work that provides interesting contrasts—although it concerns the different phenomenon of induced resistance. Many have said that, in order to induce tumor immunity, cells must be living. Some have even said that the cells must grow. In keeping with this idea, we have shown that, if red cells are broken up into a fine suspension by relatively gentle means, they lose all power to induce resistance. On the other hand, we have published convincing evidence that red-cell stromata may be as effective as red cells in inducing resistance to the subsequent implantation of tumor. I do not think that it would be profitable to attempt to get agreement on a definition of the term “living,” but washed red-cell stromata do not seem to me to be living in the usual sense of that word, and surely such cells do not grow after injection into a foreign host.

As a third and last point to illustrate the elusive character and contradictions in this field, I should like to discuss the effect of a prior inoculation of homologous blood. Following Bashford, we have found that, in mice, homologous blood has great power to induce immunity to the implantation of tumor, a tissue that presumably has considerable inherent ability to overcome minor degrees of resistance. On the other hand, Medawar has reported that erythrocytes have little or no power in rabbits to induce resistance against homografts of skin, a tissue that presumably has little capacity for overcoming resistance. Billingham has just now reported the observation of enhanced survival of skin following the injection of blood. Breyere, at the University of Maryland, College Park, Md., has recently reinvestigated this problem, using pen-bred rats. He found that a prior injection of washed erythrocytes reduced the survival time of skin homografts in rats. Furthermore, there was an interesting difference in the appearance of the specific skin graft as compared to the nonspecific homograft and to the autograft.

In conclusion, we are frequently confronted with antinomies in this field. Many of them are based upon work that appears sound, and one would be reluctant to contradict them. The difficulty arises when we try to bring them all together. This suggests to me that either there may be something wrong with the manner in which we bring the facts together, or that some of the facts may be only apparent facts. Perhaps at this stage our viewpoint may be faulty, or some critical part of the puzzle may be missing. Perhaps we are attempting a too simple or too direct transfer of available concepts from other fields into this one. It is my hope that, through the development of new concepts, we may find that what now appear as contradictions are really only paradoxes. Therein lies the hope for practical future application of today's theory.

Reference

1. WEAVER, J. M., G. H. ALGIRE & R. T. PREHN. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. J. Natl. Cancer Inst. 15: 1737-1767.

QUESTION: What is the difference between "acquired tolerance" and "enhancement"?

RUPERT BILLINGHAM: Enhancement, or the induced acceptance of a tumor homograft in a normally resistant host, is the result of pretreatment of immunologically mature animals with desiccates or saline homogenates prepared from the living homologous tissue. It depends upon an active immunological response on the part of the host—by the formation of hemagglutinins—to antigens of the red-cell type present in the inocula. From a transplantation immunity point of view, the antigenic stimulus responsible for enhancement is incomplete. It does not comprise living cells and does not result in a shortening of the lifetime of the tumor homografts subsequently transplanted.

Tolerance, on the other hand, is the induced state of specific nonreactivity towards tissue homografts that results from the exposure of embryos or very young animals to living homologous tissue cells before the awakening of their immunological response mechanism. It has been found that only those tissue inocula that are capable of eliciting transplantation immunity in adult animals are capable of conferring tolerance when inoculated into embryos. Tolerance, therefore, is the outcome of exposing immature animals to a fully antigenic stimulus. Red cells, which are incapable of eliciting transplantation immunity in the adult, are incapable of inducing tolerance in embryos, and similar results have been obtained when embryos have been inoculated with tissue preparations that would have "enhanced" the growth of homografts in adult animals. Indeed, evidence has been obtained from experiments carried out on birds that in completely tolerant animals there is complete inhibition of hemagglutinin production (a necessary condition for enhancement), despite the continued presence of an effective antigenic stimulus.

In summary, enhancement depends upon a particular type of active immune response on the part of the host, whereas tolerance depends upon a specific suppression of an animal's capacity to respond to the antigens responsible for bringing the tolerant state into being. A final, practical difference between tolerance and enhancement would appear to be one of degree of abrogation of the host's capacity to respond to the challenging homologous tissue graft. Tolerance may be virtually complete, irrespective of the type of tissue graft employed, but enhancement appears to be complete only if tumors are used as the test system. When skin homografts were used, we succeeded in prolonging their survival for only a few days.

QUESTION: What is meant by adoptive immunity?

RUPERT BILLINGHAM: Actively acquired immunity is the state of heightened resistance or sensitivity that is called into being when an animal is grafted or inoculated with homologous tissue cells.

For example, if a CBA-strain mouse is grafted with skin from an A-strain donor, it will react against its graft, causing it to break down within about 11 days. Because of its heightened resistance, regression of a second A-strain

graft subsequently transplanted to this mouse will be complete within about 6 days.

If the regional or draining lymph nodes are excised from such an actively immunized CBA mouse (primary host) and implanted intraperitoneally into a normal CBA mouse (secondary host), then the latter will behave toward an A-strain skin graft exactly as if it had itself been actively immunized. The state of immunity which it manifests is the result of the transfer of immunologically activated node cells that become incorporated in its body and continue to function just as they would have done if left undisturbed in the primary host. That transplantation immunity could be transferred in this way was first demonstrated by N. A. Mitchison.

The immunity thus created is said to be *adoptively acquired*. It may be added that neither killed lymph nodes, nor massive doses of whole blood, blood leukocytes, or serum have the power to transfer immunity to secondary hosts. Such facts argue against the supposition that the immunity acquired by the secondary hosts is due to the passive transfer of preformed antibodies. Evidence that it is not due to active immunization of the secondary hosts by antigenic material carried over in the lymph nodes transferred from the primary hosts has been presented by Billingham, Brent, and Medawar, as well as by Mitchison.

Adoptive immunity is passive in the sense that it is of second-hand origin, but it is active in the sense that it depends upon the introduction and continued functioning of immunologically activated cells. A distinction between adoptively and passively immunized animals is that the former are capable of a secondary response. They differ from actively immunized animals in that they have never given a primary response.

QUESTION: What is the difference between a tumor homograft and that of a normal tissue, such as skin?

RUPERT BILLINGHAM: To the best of my knowledge, no one has ever made a strict comparison of the fates of skin and tumor homografts transplanted between animals of the same inbred strains. There is a sufficient abundance of literature, however, dealing with homografts of the one type or the other to enable one to answer the question with a considerable degree of confidence.

The fact that the genetic laws of transplantation, worked out by Little and Snell using tumor homografts, seem to apply equally well in the case of homotransplants of skin suggests that, fundamentally, there is little difference between them. Furthermore, Mitchison's accurate estimates of the survival of a lymphosarcoma transplanted between mouse strains of distant genetic affinity have given results comparable to those obtained for skin transplanted between members of mouse strains of comparable genetic disparity.

The main difference between tumor homografts and those of skin is that the former, probably because of their higher intrinsically determined growth rate, once established, appear to be able to withstand a degree of immunological opposition that would prove fatal to a skin homograft. This does not mean, of course, that tumor homografts are necessarily less effective in eliciting transplantation immunity. It means only that their susceptibility is less. As Medawar has pointed out, since tumors seem to need comparatively little en-

couragement before they will grow in genetically alien soil, they may therefore exaggerate the efficacy of treatments designed to promote their well-being.

Again, it must be emphasized that this difference between tumors and skin, with respect to their capacity to withstand immunological opposition, is only one of degree. Few tumors can be transplanted between mouse strains when the genetic disparity is very great. Evidence suggesting that the difference is not due to a specific property of tumors is derived from the fact that ovarian homografts, and probably those of at least some other endocrine tissues, are less exacting than skin with respect to their immunological requirements for successful homotransplantation.

One property that at present appears to distinguish tumor homografts from those of normal tissues is their capacity to undergo antigenic changes, as revealed in an increased acceptability to hitherto resistant hosts. The work of Barrett and Deringer suggests that such changes may not always be the outcome of growing the tumor cells in the face of a weak immunological opposition, though this is the situation that most commonly appears to result in antigenic changes. They demonstrated that a transplantable tumor indigenous to one inbred strain, after a single passage through the F_1 generation between that strain and a resistant one, shows a permanently increased transplantability to the F_2 or resistant back-cross mice. Whether these various changes represent transformations, adaptations, or selections from variants present in enormous cell populations need not concern us here. I merely wish to draw attention to the fact that tumors do undergo antigenic changes as a consequence of homotransplantation and that, so far, there is no evidence that normal tissues show similar tendencies. It must be admitted that it would be technically rather difficult to study homografts of normal tissues with this point in mind, because they do not normally increase in amount after grafting.

There is one anatomical difference between an orthotopic skin homograft and a subcutaneously implanted tumor homograft that may be responsible for a difference in the expression of an immune state, once it has been called into being. Orthotopic skin grafts are vascularized fairly rapidly as a result of some sort of anastomosis between their own vessels and those of the host in the graft bed and, consequently, there is minimal nonspecific necrosis. In the case of subcutaneous tumor implants, by contrast, there is probably a high degree of nonspecific necrosis because of the poor vascularity of the environment into which they are introduced and the consequent delay in vascularization. The palpable tumor that may rapidly arise is therefore the product of the high rate of proliferative growth of comparatively few cells that survived in the initial inoculum. This fact suggests very strongly that the vessels in an established tumor homograft, unlike those in a skin homograft, are of *host* origin.

THE HOMOTRANSPLANTATION OF FUNCTIONAL ERYTHROPOIETIC ELEMENTS IN THE RAT FOLLOWING TOTAL-BODY IRRADIATION

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The barrier to complete interchangeability of normal, functional tissues between animals is generally considered to be immunogenetic in the sense that the genotype of the individual determines the antigenic phenotype of his tissues. It is well established that the probability of success in establishment of a tissue transplant is a function of the coefficient of relationship between the donor and the recipient. In normal situations the probability of a successful transplant is effectively zero in the absence of genetic and therefore antigenic identity between host and donor.

It has been found that under certain circumstances the strict genetic requirements for successful homotransplantation can be circumvented. The observations of Owen (1945) on the blood types of dizygotic twins in cattle and a similar observation in man (Dunsford *et al.*, 1953) have demonstrated that the fetus will accept a natural homograft of erythropoietic tissue from its twin, whose tissues it would certainly reject as an adult. Artificial homotransplantation of erythropoietic tissue into fetal rats has been demonstrated by Ripley and Owen (see Horowitz and Owen, 1954). Thus the use of a fetal host circumvents, at least in some cases, the immunogenetic restrictions to homotransplantation. It has been further demonstrated that establishment of a homograft in a fetal host renders the host tolerant of skin grafts from the same donor in later life. This was first shown for fraternal twins in cattle (Anderson *et al.*, 1951; and Billingham *et al.*, 1952). It was also demonstrated in both chickens and mice (Billingham *et al.*, 1953, 1955) that injection (and probably implantation) of adult cells into an embryonic host renders the host subsequently tolerant of homografts from the same donor.

The mentioned procedures, which require access to the host during fetal life, raised the question of whether the importance of the genetic relation between host and homograft donor could be decreased by treatment of an adult host. Main and Prehn (1955) found that the skin of mice of 1 strain could be successfully grafted onto adult hosts of another strain if the latter had been irradiated heavily and injected, immediately postirradiation, with bone marrow from a hybrid of the 2 strains. Similarly, it has been demonstrated that homologous rat bone marrow can be successfully transplanted as a functional erythropoietic tissue following total-body X irradiation of the recipients (Lindsley *et al.*, 1955), whereas nonirradiated hosts fail to accept such a transplant.

* Under Contract No. W-7405-eng-26 for the Atomic Energy Commission, Washington, D. C.

† Aided by Contract Nonr-220(20) between the Office of Naval Research, Department of the Navy, and the California Institute of Technology.

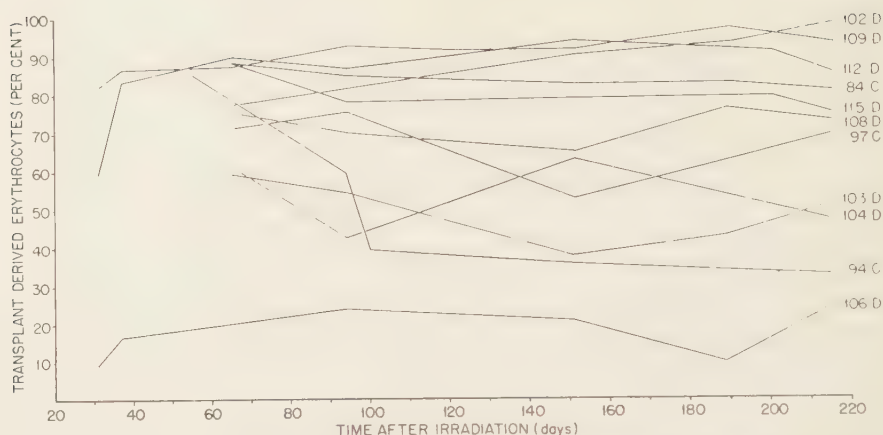


FIGURE 1. Proportion of erythrocytes derived from bone marrow transplanted into animals 18 hours after 750 r of whole-body X radiation, as measured between the 30th and 215th days after irradiation.

The transplanted marrow becomes permanently established and contributes a major portion of the peripheral erythrocyte population. Of 17 animals that survived the irradiation for at least 20 days, 15 gave evidence of erythrocytes contributed by the transplant. Quantitative estimates of the composition of the peripheral erythrocyte population reveal that 10 to 95 per cent of the circulating red cells were derived from the transplanted marrow (FIGURE 1). Estimates on individual animals made between the 35th and 215th days post-irradiation are in good agreement, if allowance is made for the expected counting error, except in animal 94 C, in which there was evidence of a gradual decrease in the number of erythrocytes derived from the transplant. Another animal, not shown in FIGURE 1, had 90 to 95 per cent transplant-derived erythrocytes on days 94, 100, 112, and 147, but only 65 per cent when next tested on the 260th day after irradiation.

The following experiments were undertaken to follow the increase in donor erythrocytes from the time of transplantation until equilibrium is reached, and to relate such increases to the changes in the erythrocyte count induced by irradiation.

Materials and Methods

Animals. In rats, a pair of alleles determines the existence of a pair of cellular antigens, C and D. The 2 homozygous types, C/C and D/D, carry antigens C and D respectively on their erythrocytes. Heterozygous animals, C/D, carry both antigens C and D on their erythrocytes (Owen, 1948). A C/C line and a D/D line were started from the progeny of heterozygotes derived from backcrosses of the F_1 from a cross of an inbred (47 generations) Irish strain (D/D) crossed with another line inbred for nine generations (C/C). No attempt at inbreeding has been made. Consequently, both lines are almost certainly segregating for a number of antigenic differences as they are for several coat color factors.

Antisera. Antibodies were prepared by injecting 0.5 ml. of a 20 per cent red cell suspension from one homozygous line into the ear vein of a rabbit 3 times a week for 3 weeks. Serum was collected within a week to 10 days following the last injection, incubated at 56° C. for 45 minutes, diluted 1:4 with saline, and absorbed 4 times with an equal volume of pooled erythrocytes from rats of the other homozygous line. The titers of the absorbed sera that would give complete (95 to 100 per cent) agglutination of homologous cells suspended at a concentration of $12 \text{ to } 14 \times 10^8 \text{ mm.}^3$ in the diluted sera ranged from 1:2 to 1:16. Satisfactory anti-C serum was easily obtained, but some difficulty has been encountered in obtaining anti-D serum with a sufficiently high titer for quantitative work.

Treatment of animals. For irradiation, animals were placed in an 8-sectored, shallow, cylindrical lucite container mounted on a rotating table with a target-object distance of 93.7 cm. X rays were from a 250-kv., General Electric Maxitron at 30 mAmp. with 3 mm. of aluminum filtration. The animals were irradiated with 700 r delivered at a rate of about 55r/min. This dose was estimated to be significantly below the LD₅₀/30 days, judging from experience with previous generations from the same lines. Approximately 18 hours after irradiation, each animal was injected with bone marrow from a rat of the opposite genetic type. Each donor was decapitated and the femora immediately excised. Both ends of a femur were removed, and the marrow plug was forced out with air pressure. The marrow was suspended in Tyrode's solution or saline and broken up into single cells and small cellular aggregates by forcing through 19-, 23-, and then 26-gauge needles. The marrow of 2 femora suspended in 0.5 ml. was injected into the jugular vein of each recipient.

Estimation of relative numbers of host-derived and transplant-derived erythrocytes. The cellular composition of the peripheral erythrocyte population of the irradiated animals was estimated from samples of blood withdrawn from the tail at various intervals following treatment. The method used was adapted from a technique worked out by Wilkie and Becker for quantitative determination of hemagglutinin titers (1955). Blood was taken from the tail into a red cell pipet, and hemocytometer counts were made. Several drops of blood were then collected in 2.5 per cent sodium citrate in 0.5 per cent NaCl, washed once in 100 volumes of PO₄ buffer, and diluted in PO₄ buffer to about $3 \times 10^4 \text{ cells/mm.}^3$. One hundred $\mu\text{l.}$ aliquots of the cell suspension were added to (1) 200 $\mu\text{l.}$ anti-C serum, (2) 200 $\mu\text{l.}$ of 8 parts isotonic PO₄ buffer to 1 part normal rat serum, and (3) 200 $\mu\text{l.}$ anti-D serum. The tubes were allowed to stand for 10 minutes and were then centrifuged lightly for 2 minutes to form a loose pellet at the bottom of the tube. This aggregate was gently resuspended, and the tubes were placed on a Kahn shaker and agitated at approximately 120 oscillations per minute for 2 hours. The contents of each tube were thoroughly resuspended (cells settle out when agitated at 120 oscillations per minute), a sample was placed in a hemocytometer chamber, and the unagglutinated cells were counted. The antisera were used in concentrations sufficient to effect complete agglutination of homologous cells at $12 \text{ to } 14 \times 10^3 \text{ cells/mm.}^3$, the same concentration as used in the reaction tubes. Accordingly, all cells carrying the homologous antigen should have been agglutinated

in the reaction tubes. The unagglutinated cells in the tube containing anti-C serum were those that had no C antigen and were therefore derived from precursors of genotype D/D. Similarly, unagglutinated cells in the tube containing anti-D were derived from precursors of genotype C/C. The PO_4 buffer tube provided a measure of the concentration of the original red cell suspension. Ideally, the sum of the free cells in the 2 tubes containing the antisera should equal the number of cells in the PO_4 buffer tube. This ideal may be realized when both antisera are available and reliable. In the present series of experiments, however, the behavior of anti-D was erratic, and the determinations were made on the basis of the anti-C and PO_4 buffer tubes alone. This, unhappily, deprives the estimates of the independent check provided by the anti-D tube. Activity of the serum was checked against both C and D cells before each set of measurements. The proportion of transplant-derived erythrocytes estimated from the free-cell counts was multiplied by the total red blood count to provide an estimate of the number of transplant-derived erythrocytes/mm.³

Results

Changes in erythrocyte composition of 20 animals observed for 49 days following irradiation and injection with homologous bone marrow are summarized in TABLE 1. Since anti-C serum was used to differentiate between the C and D erythrocytes, the transplant-derived erythrocytes were agglutinated in samples from type-D animals, while the host-derived erythrocytes were agglutinated in samples from type-C animals. In samples from D recipients where no agglutination was observed, the free cell counts of the antiserum and control tubes were not always exactly equal, resulting in values other than zero for the estimated number of implant-derived cells. These cases (footnote ‡ in TABLE 1) provide some estimate of the errors of the method. Furthermore, since the free cells in samples from type-D animals were host-derived, the implant-derived cells were estimated by subtracting the number of free cells in the antiserum tube from those in the control tube. Where the number of transplant-derived erythrocytes (agglutinated cells) was small, there was occasionally a negative estimate of their number.

It can be seen in the table that evidence of transplant-derived erythrocytes usually appears on the eighth day after irradiation. The subsequent changes in erythrocyte composition of the animals are variable, with proportions of 0 to 95 per cent implant-derived erythrocytes becoming established, presumably to be maintained as in the animals shown in FIGURE 1. Therefore, these animals are mosaics, with 2 types of bone marrow, each elaborating mature peripheral red blood cells, and neither affecting the other in such a way as to alter the composition of the circulating red cell population. The results from 8 representative animals from TABLE 1 are plotted in FIGURE 2.

The variation in number of transplant-derived erythrocytes within animals during the experiment, and the variation between animals at selected times, were calculated by an analysis of variance. The animals surviving until the 49th day postirradiation could be divided into 2 groups, those with unsuccessful

TABLE 1
CHANGES IN ERYTHROCYTE COMPOSITION OF THE CIRCULATING BLOOD IN ANIMALS GIVEN HOMOLOGOUS BONE-MARROW INJECTIONS 18 HOURS AFTER 700 R OF WHOLE-BODY X RADIATION

Animal No. & sex	No. of transplant-derived cells/total erythrocyte count on indicated day (10^{-6} cells/mm. ²)									
	0	8*	11	15	22	29	35	42	49	
171 C ♀	0 7.65	+ 8.30	0.75 6.75	1 60 8.00	2 95 7.10	4 15 9.00	5.35 7.60	6 20 8.25	7.05 7.85	
172 C ♀	0 9.10	+ 8.15	1.05 7.50	2 00 9.00	3 40 8.90	4 80 8.50	3.90 5.75	6.15 6.90	5.55 6.35	
173 C ♀	0 10.00	+ 8.95	0.15 7.85	0.10 6.90	0.15 8.00	0.15 8.60	0.10 7.85	0.10 7.65	0.15 8.85	
174 C ♀	0 9.90	+ 7.75	1.30/6.40†							
175 D ♀	0 9.70	± 6.60	0.45 7.00	1 10 6.65	1 20 6.10	0.10 7.35	0.15 7.35	1 40 6.50	1.50 7.10	
176 D ♀	0 8.95	± 7.70	0.80† 6.15	0 05 4.65	0.10 6.35	1.00 7.45	0.65† 6.20	0.45 7.45	0.55 5.25	
177 D ♀	0 7.65	+ 5.05	0.75 5.00	2.10 5.60	4.80 8.15	6.05 8.60	6.40 8.30	7.20 8.85	6.85 8.15	
178 D ♀	0 8.80	0 8.30	0.55† 6.90	1.10 4.85	1.20† 7.20	0.30† 7.20	0.25† 6.20	0† 7.35	0.35† 8.15	
179 D ♂	0 7.95	± 7.90	0.60† 9.15	— 1.20/6.45	0.40† 6.75†	1.65 9.25	5.95 9.20	5.00 8.60	6.10 9.00	
180 D ♂	0 8.70	± 8.70	1.30 6.95	1.15 7.10	1.80 7.60	0.45/1.70†				
181 D ♂	0 9.70	± 8.50	— 0.15/4.30	0.15 4.30	0.20† 4.60	1.10 8.40	4.15 8.75	4.10 7.90	5.15 8.35	
182 D ♂	0 10.65	+ 8.70	2.05 6.60	2.05 8.20	3.80 8.00					
183 D ♂	0 7.75	0 7.45	0 35 8.60	— 0.10 7.55	0.60 7.95	0.60/7.30	3.35 8.50	3.60 8.60	5.25 9.45	
184 D ♂	0 8.30	0 7.75	1.30 8.00	1.00 8.60	1.70 6.95	1.45/7.50§				
185 D ♂	0 10.30	0 9.40	2.15/13.00	1.60/8.15	2.80/7.40§					
186 D ♂	0 10.15	0 9.35	1.30 9.45	1.45 8.15	2.65 9.10	2.40 9.20	3.45 7.90	3.95 9.30	3.75 7.60	
187 D ♀	0 8.90	0 6.10	0.50 6.60	1.15 5.00	— 0.15 5.70	0.50† 5.50	0.55 4.20	1.40 4.90	— 0.30† 7.75	
188 D ♀	0 8.70	0 8.45	— 0.20 6.20	1.40 6.80	2.55 7.45	3.90 9.65	5.65 8.95§			
189 D ♀	0 9.70	+ 9.05	2.05 8.00	2.00 7.70	1.80 7.55	4.15 8.80	4.25 7.60	4.00 8.05	5.25 8.50	
190 D ♀	0 6.90	0 7.95	1.25† 7.85	0.15† 6.35	0.40† 6.40	0.35† 7.40	1.45† 6.90	1.30 6.95	0.50† 6.85	

* Presence or absence of transplant-derived erythrocytes determined microscopically but proportion not estimated.

† Acute irradiation death before next determination.

‡ Numerators represent deviations from zero (see text).

§ Death without symptoms of acute irradiation before next determination.

ful and those with successful transplants. Animals that showed few or no transplant-derived erythrocytes had values homogeneous among animals and among samples taken from those animals at different times after irradiation (173 C, 175 D, 176 D, 178 D, 187 D, and 190 D). The second group was het-

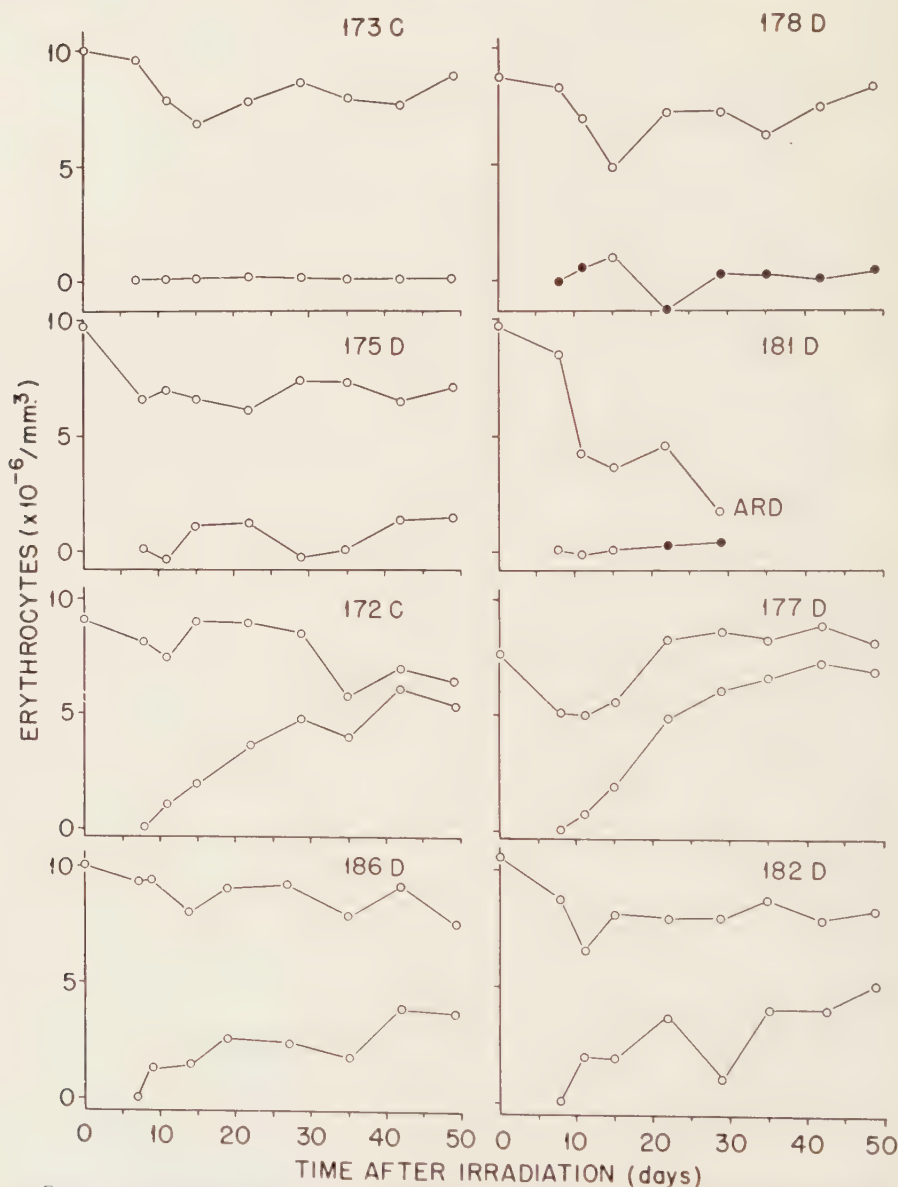


FIGURE 2. Changes in estimated erythrocyte composition in representative animals from TABLE 1. The upper curve in each case represents the total erythrocyte count, and the lower curve is the estimated number of transplant-derived erythrocytes. Solid circles represent estimates footnoted 'b' in TABLE 1 (see text). ARD = acute radiation death.

erogeneous among animals in regard to the number of transplant-derived erythrocytes, and showed a definite increase with time in the number of foreign cells (171 C, 172 C, 177 D, 180 D, 182 D, 183 D, 186 D, and 189 D).

The contribution of interanimal variation and temporal variation to the total variance of the total erythrocyte count of each of these groups was then determined. Each group showed heterogeneity among animals ($p < 0.001$ for the unsuccessfully transplanted animals and $p = 0.01$ – 0.05 for the successfully transplanted animals). With respect to day-to-day variation, the unsuccessfully transplanted animals showed highly significant differences in red cell count ($p < 0.001$), whereas the difference among the successfully transplanted animals was not significant at the 5 per cent level ($p = 0.087$). The mean red blood cell counts of each group for each day were computed and the 95 per cent confidence limits established (FIGURE 3). The curves are parallel and have negative slope for the first 10 days after irradiation. Beginning at this time the successfully transplanted animals show a rapid return to the original level of the total count, whereas the cell counts of the unsuccessfully transplanted animals continue to decrease until the 15th day and then slowly approach a normal level at 50 days. It seems likely that the point of departure of the 2 curves is associated with the observed increase in transplant-derived erythrocytes, which begins on the eighth day postirradiation.

The difference between the mean red cell counts of the 2 groups of animals

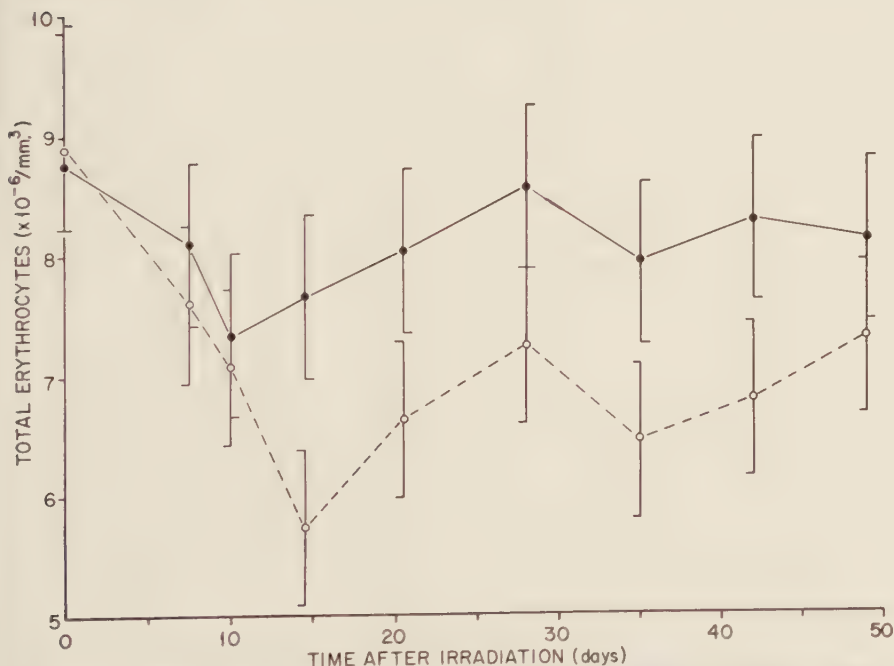


FIGURE 3. Changes in total erythrocyte count in animals following 700 r of total-body X radiation and homologous bone marrow. Solid circles represent mean red cell counts from animals with successful transplants. Open circles represent mean red cell counts from animals with unsuccessful transplants. Ninety-five per cent confidence intervals are indicated.

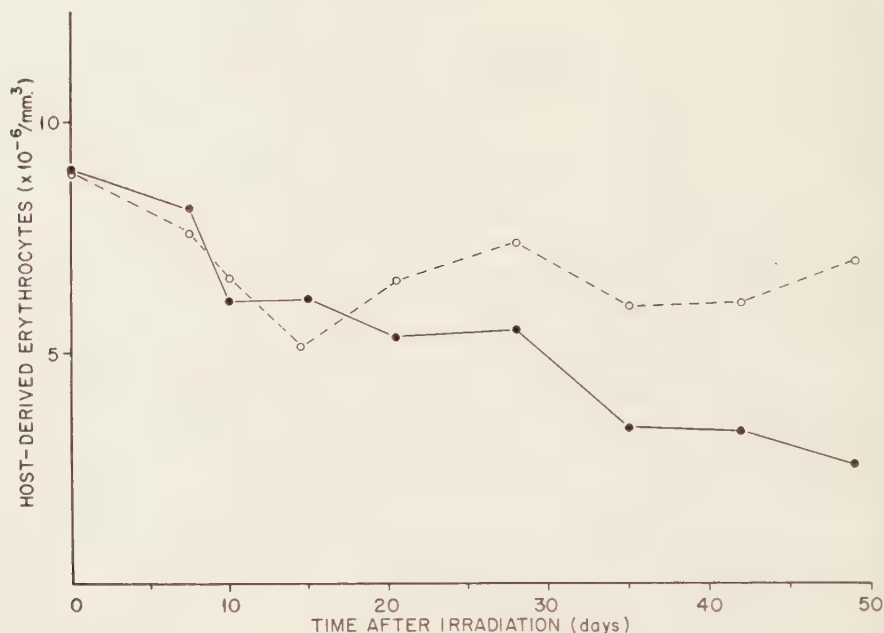


FIGURE 4. Changes in number of host-derived erythrocytes (total cells less transplant-derived cells) following 700 r of total-body X radiation and homologous bone marrow. Solid circles represent the mean number of host-derived cells from animals with successful transplants. Open circles represent mean number of host-derived cells from animals with unsuccessful transplants.

is too small to account for the contribution of the implanted marrow to the total red cell count, which suggests that successful transplantation has an adverse effect on the recipient's marrow. It appears that the irradiated marrow may be unable to compete successfully with the transplanted marrow during the period of bone-marrow recovery. This is illustrated in FIGURE 4, where the mean number of host-derived erythrocytes is plotted for successfully and unsuccessfully transplanted animals.

Discussion

Intravenous injection of homologous bone marrow into rats following total-body X irradiation of 700 or 750 r resulted in the establishment of an actively erythropoietic bone-marrow graft in well over half the animals treated. Ripley and Owen (see Horowitz and Owen, 1954), employing the same antigenic markers used in the present experiments, demonstrated that injection of fetal liver and spleen cells into the chorionic vessels of fetal rats also results in the establishment of a permanent erythropoietic graft. This similarity in reaction of fetal and irradiated rats to transplanted cells justifies a comparative inquiry into the response to transplantation in fetal and irradiated systems.

The antibody-producing capabilities of an animal that has received a homograft during fetal life are, in general, unimpaired. This has been demonstrated by studies on skin grafting in the naturally produced erythrocyte mosaics

found in fraternal twins in cattle (Anderson *et al.*, 1951; and Billingham *et al.*, 1952). It has been shown that an animal that is mosaic for erythrocytes from his twin is partially or completely tolerant of a skin graft from that twin, but rejects in normal fashion a skin graft from any other source, including other full sibs and mother. The postpartum existence in the recipient of a very specific acquired tolerance of the constellation of antigens characteristic of the donor in an otherwise normally active antibody-producing system is the observed result of homotransplantation into the fetus. This observation has been confirmed in chickens injected as embryos and in mice injected as fetuses (Billingham *et al.*, 1954), and in rats injected as newborn animals with homologous adult cells (Woodruff and Simpson, 1955). It has also been observed in twin chicks hatched from double yolked eggs (Brent—see Brambell, 1955) and in chicks whose embryonic circulations were joined through egg parabiosis (Hašek, 1953). (Persistence of the original cellular inoculum has been demonstrated only in twin chickens.)

A particularly effective demonstration of the specificity of inactivation of the immature system involves a soluble antigen, human albumin. Cinader and Dubert (1955) have shown that injection of human albumin into newborn rabbits, in amounts shown to be actively immunizing if injected 8 weeks later, prevented those rabbits from forming antibodies in response to subsequent injections of human albumin. Subsequent injections of human albumin to which benzene *p*-sulfonic acid had been diazotized did, however, elicit antibody formation. Investigation of these antibodies proved them to be directed primarily against the diazo group, with little or no reaction with the human albumin alone.

The antibody-producing capabilities of animals that have accepted homografts following irradiation have not been thoroughly investigated. The work of Main and Prehn (1955) may indicate, however, that the inhibition is less specific than in the fetally homografted individual. Mice of inbred strain A were irradiated with 800 to 850 r and immediately injected with bone marrow of F₁ mice from an A × B cross. Twenty-four to 30 days later these mice were challenged with a skin graft from inbred strain-B mice. Grafts became established in 33 of 36 animals challenged. Two of 31 irradiated strain-A mice injected with strain-A bone marrow failed to reject strain-B skin grafts when similarly challenged. An adequate irradiated control could not be performed since the dose of radiation used does not permit survival in the absence of bone-marrow transfusion. A dose comparable with respect to survival, 350 r, did not permit homologous skin grafting 24 to 30 days after irradiation. The question of persistence of the transfused marrow was not examined in these experiments, although it was suggested that the introduced marrow probably became established.

The ability of the original grafted cells to evoke antibody formation has not been investigated in either fetal or irradiated homografted animals. That skin homografted onto mice made tolerant by fetal injection with cells from the donor strain does not lose its antigenic powers has, however, been demonstrated (Billingham *et al.*, 1955), but it has not been determined whether the fetally-administered donor strain cells persist. Mice of strain A injected as

fetuses with cells from mice of strain B will, after birth, accept skin grafts from strain-B donors. Intraperitoneal introduction of chopped lymph nodes from an animal of strain A into a tolerant A animal that has an established strain-B skin graft causes a normal incompatibility reaction and rejection of the skin graft in 15 to 23 days. More rapid rejection of the skin graft can be achieved by use of lymph nodes from an A-strain mouse that has previously rejected a B-strain skin graft. No experiments of this type have been carried out on postirradiation homografted tissues.

The immune mechanisms of the embryonic or fetal animal and of the post-irradiation animal are known to function submaximally. The observations on homotransplantation indicate that the development of the antibody-producing system goes through 3 stages. First there is a period during which homologous cells are accepted and become established. It is during this time that acquired tolerance may be induced. Then there is a period in which cells are neither accepted nor rejected, followed by a period during which homologous cells elicit an immune response and are actively rejected. The relation of parturition (or hatching, in birds) to these periods varies with the species. Titration of circulating antibody in animals injected with antigens following irradiation shows that the immediate effect of a moderate to large dose of whole-body irradiation is the partial or complete destruction of an animal's ability to produce antibodies (Craddock and Lawrence, 1948; Kohn, 1951; Dixon *et al.*, 1952; Taliaferro and Taliaferro, 1954; and Smith and Ruth, 1955). The ability of the mouse to produce sheep hemolysin begins to return 4 weeks following 450 r of radiation and becomes appreciable during the fifth, sixth, and seventh weeks postirradiation (Smith and Ruth, 1955). Whether the return of the immune mechanism following irradiation goes through the same 3 stages of reaction to homotransplantation as the original development of the system has not been adequately investigated.

The foregoing discussion has been a consideration of the response of an animal to an antigen as a function of the effective potential of its immune system. Felton (1949) has shown that an animal's response is also a function of the amount of antigen it receives. He and his co-workers have shown that, although 0.5 μ g. of pneumococcus polysaccharide represents an immunizing dose and will render a mouse resistant to the strain of pneumococcus from which the polysaccharide was prepared, 5.0 μ g. 0.5 mg. is not immunizing but will, in fact, prevent a mouse from becoming immunized by a subsequent immunizing dose given as long as 15 months after the massive dose. This phenomenon has been termed by Felton "immunological paralysis," and it has been shown to be type specific (Felton *et al.*, 1955).

The similarity between immunological paralysis and acquired tolerance tempts one to speculate that the reaction of an animal to an antigen, for example a tissue homograft, is a function of the amount of the inoculum and the effective potential of the immune system. This function would be such that, in the region of antigen excess, the probability of a successful homograft could be increased by increasing the size of the inoculum or decreasing the potential of the immune system.

Most studies of the influence of X irradiation on homotransplantation have

involved the use of tumors. In some cases they demonstrate induced lowered resistance to transplanted tumor tissue (Toolan, 1953). Homotransplantation of normal tissue onto an irradiated host has rarely been reported to yield a permanently functioning graft. Dempster and his associates (1950) found increased, but never complete, survival of skin homografted onto rabbits 24 hours after 250 r. Rabinovici (1947) found no effect of 500 r on survival of skin homografted onto rats 24 hours after irradiation. Main and Prehn (1955) found no survival of skin homografted to mice 24 to 30 days after 350 r, and 6.5 per cent survival 24 to 30 days after 800 to 850 r and isologous marrow. Hardin and Werder (1954, 1955), however, have reported permanently successful skin homografting in 61 per cent of mice grafted 24 hours after receiving 300 r. They also report 32.5 per cent successful skin homografts from irradiated donors (300 r) to unirradiated recipients. This observation seems difficult to interpret on the basis of an immune response. Makinodan (1956) has observed, in mice given rat bone marrow following 950 r of radiation, the establishment of mosaics containing erythrocytes derived from the transplant alone.

These homograft attempts have not been carried out under identical conditions. Consequently, it is difficult to evaluate the implications of success in one instance and failure in another. The variables that might affect the fate of tissue homotransplanted after total-body irradiation should be considered: the possible influence of time of graft in relation to the time of irradiation, the dose of radiation, the antigenicity of the graft, and the dose of grafted cells.

The observed duration of the effect of fairly large doses of X radiation on the immune system (Craddock and Lawrence, 1948; Taliaferro and Taliaferro, 1954; and Smith and Ruth, 1955) indicates that the best time to attempt transplantation is shortly after irradiation, during the period of maximum inhibition of antibody response. The magnitude and extent in time of inhibition may be expected to be dose-dependent, in such a way that the activity of the antibody-producing system is decreased more and for a longer period of time with higher doses. As Main and Prehn (1955) pointed out, such a relation might be responsible for their ability to obtain a few successful skin grafts following 800 r and isologous marrow, but none following 350 r. This may mean that the previous suggestion, based on this observation, that injection of homologous cells following irradiation induces an acquired tolerance that is less specific than that resulting from embryonic transplantation, is spurious. Rabinovici's failure (1947) to find any effect of 500 r on the fate of skin homografted onto rats 24 hours after irradiation is hard to reconcile with the delayed rejection of skin homografted to rabbits 24 hours after a dose of only 250 r (Dempster *et al.*, 1950) and the acceptance of skin homografted to mice 24 hours after a dose of 300 r (Hardin and Werder, 1954, 1955).

It has been demonstrated in this paper that bone marrow can be readily transplanted into rats 18 hours after a dose of 700 r. The difference in transplantation results with skin and bone marrow in the rat may reflect the different radiation doses used, or a greater genetic difference between host and donor in the skin transplant experiments than in the bone-marrow transplant experiments. It may, however, represent a true difference in transplantability of

skin and marrow. The antigenicity of the skin may be different from that of the bone marrow, that is, skin may be characterized by a greater array of antigenic sites than the marrow and may therefore be more difficult to transplant. The tolerance to skin grafts induced by previous marrow injection in the experiments of Main and Prehn (1955), however, are not consistent with such a hypothesis. Another possibility is that the antigenicity of skin and marrow is quantitatively different, perhaps more because of the general distribution of a bone-marrow inoculum than the actual number of cells transplanted. The work of Billingham *et al.* (1954) on the origin of adoptively acquired immunity to skin homografts has demonstrated that an animal's immune response to a skin homograft is localized, and that production of antibody can be demonstrated only for lymph nodes from the immediate vicinity of the graft. It may be, therefore, because of the general distribution of injected bone marrow throughout the system, that it represents, cell for cell, a much more massive dose of antigen than a skin graft. Thus it may follow that the most profitable approach to homotransplantation in adult animals would be (1) to inactivate the immune response to an extent that would allow (2) transfusion of some actively proliferating cell suspension such as bone marrow. If this transplant becomes established, then (3) the animal should be tolerant of additional tissue transplants from the same source as the bone marrow.

Summary

Persistent homotransplantation of bone marrow has been obtained in rats by intravenous injection of bone-marrow suspensions after relatively large doses of whole-body X radiation. The transplant has been followed by differential agglutination of the red cells from the peripheral circulation for an antigenic difference existing between the host and donor red cells. Foreign cells first appear in the circulation 8 days following irradiation, and by 50 days they comprise 0 to 95 per cent of the circulating red cell population. They are then apparently maintained indefinitely at this level. The total erythrocyte counts in the group of rats that did not have successful bone-marrow implants after irradiation fell to a lower level and returned toward normal more slowly than in the group having successful transplants. The quantitative aspects of these counts suggest that the irradiated host marrow cannot compete successfully with the implanted marrow.

Acknowledgment

The authors wish to express their appreciation to A. W. Kimball and G. J. Atta for their help in the statistical analysis of the data.

References

- ANDERSON, D., R. E. BILLINGHAM, G. H. LAMPKIN & P. B. MEDAWAR. 1951. The use of skin grafting to distinguish between monozygotic and dizygotic twins in cattle. *Heredity*, **5**: 379-397.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature*, **172**: 603-606.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1954. Quantitative studies on tissue

- transplantation immunity. II. The origin, strength, and duration of actively and adoptively acquired immunity. *Proc. Roy. Soc. London*. **B143**: 58-80.
- BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1955. Acquired tolerance of skin homografts. *Ann. N. Y. Acad. Sci.* **59**(3): 409-416.
- BILLINGHAM, R. E., G. H. LAMPKIN, P. B. MEDAWAR & H. L. WILLIAMS. 1952. Tolerance to homografts, twin diagnosis, and the freemartin condition in cattle. *Heredity*. **6**: 201-212.
- BRAMBELL, F. W. R. 1955. Immunity and development. *Advancement of Sci.* **12**: 177-187.
- CINADER, B. & J. M. DUBERT. 1955. Acquired immune tolerance to human albumin and the response to subsequent injections of diazo human albumin. *Brit. J. Exptl. Pathol.* **36**: 515-529.
- CRADDOCK, C. G., JR. & J. S. LAWRENCE. 1948. The effect of roentgen irradiation on antibody formation in rabbits. *J. Immunol.* **60**: 241-254.
- DEMPSTER, W. J., B. LENNON & J. W. BOAG. 1950. Prolongation of survival of skin homografts in the rabbit by irradiation of the host. *Brit. J. Exptl. Pathol.* **31**: 670-679.
- DIXON, F. J., D. W. TALMAGE & P. H. MAURER. 1952. Radiosensitive and radioresistant phases in the antibody response. *J. Immunol.* **68**: 693-700.
- DUNSFORD, I., C. C. BOWLEY, A. M. HUTCHISON, J. S. THOMPSON, R. SAGER & R. R. RACE. 1953. A human blood-group chimera. *Brit. Med. J.* **4827**: 81.
- FELTON, L. D. 1949. The significance of antigen in animal tissues. *J. Immunol.* **61**: 107-117.
- FELTON, L. D., G. KAUFFMANN, B. PRESCOTT & B. OTTINGER. 1955. Studies on the mechanism of immunological paralysis induced in mice by pneumococcal polysaccharides. *J. Immunol.* **74**: 17-26.
- HARDIN, C. A. & A. A. WERDER. 1954. The effect of total body irradiation on the survival of homologous skin grafts on CFW mice. *Plastic and Reconstr. Surg.* **13**: 40-45.
- HARDIN, C. A. & A. A. WERDER. 1955. A one year study of surviving homografted mouse skin. *Plastic and Reconstr. Surg.* **15**: 107-113.
- HÁSEK, M. 1953. Vegetative hybridization of animals by connecting their blood streams during embryological development. (In Czech.) *Ceskoslov. Biol.* **2**: 265-277.
- HOROWITZ, N. H. & R. D. OWEN. 1954. Physiological aspects of genetics. *Ann. Rev. Physiol.* **16**: 81-102.
- KOHN, H. I. 1951. Effect of X rays upon hemolysin production in the rat. *J. Immunol.* **66**: 525-533.
- LINDSLEY, D. L., T. T. ODELL, JR. & F. G. TAUSCHE. 1955. Implantation of functional erythropoietic elements following total body irradiation. *Proc. Soc. Exptl. Biol. Med.* **90**: 512-515.
- MAIN, J. M. & R. T. PREHN. 1955. Successful skin homografts after the administration of high dosage X radiation and homologous bone marrow. *J. Natl. Cancer Inst.* **15**: 1023-1029.
- MAKINODAN, T. 1956. Circulating rat cells in lethally irradiated mice protected with rat bone marrow. *Proc. Soc. Biol. Med.* **92**: 174-179.
- OWEN, R. D. 1945. Immunogenetic consequences of vascular anastomoses between bovine twins. *Science*. **102**: 400-401.
- OWEN, R. D. 1948. Antigenic characteristics of rat erythrocytes and their use as markers for parabiotic exchange. *Genetics*. **33**: 623-624.
- RABINOVICI, N. 1947. Fate of skin homotransplants performed on previously X rayed rats. *Plastic and Reconstr. Surg.* **2**: 413-418.
- SMITH, F. & H. J. RUTH. 1955. Hemolysin production in irradiated mice given spleen or bone-marrow homogenate. *Proc. Soc. Exptl. Biol. Med.* **90**: 187-191.
- TALLAFERRO, W. H. & L. G. TALLAFERRO. 1954. Further studies on the radiosensitive stages in hemolysin formation. *J. Infectious Diseases*. **95**: 134-141.
- TOOLAN, H. W. 1953. Conditioning of the host. *J. Natl. Cancer Inst.* **14**: 745-767.
- WILKIE, M. H. & E. L. BECKER. 1955. Quantitative studies in hemagglutination. I. Assay of anti-B isohemagglutinins. *J. Immunol.* **74**: 192-198.
- WOODRUFF, M. F. A. & L. O. SIMPSON. 1955. Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth. *Brit. J. Exptl. Pathol.* **36**: 494-499.

Discussion of the Paper

HILARY KOPROWSKI (*Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y.*): The findings of Lorenz *et al.*¹ that the administration of

bone marrow to mice exposed to the lethal effect of X irradiation increases survival time and promotes recovery gave impetus to a series of investigations in which this technique has been employed. (One of these was a study by Main and Prehn² of homologous grafting of skin following the inoculation of donor's bone marrow into an irradiated recipient. The latter authors mentioned that "the precise mechanism of action of the bone marrow in these experiments remains obscure.")

The paper by Odell *et al.* advances our knowledge concerning the mechanism by which the irradiated animal is protected. The fact that the injected bone marrow proliferates is proved beyond any doubt by the persistent mosaicism of erythrocytes in the recipient. I am also inclined to agree with Lindsley that in all probability we are not dealing here with the phenomenon of transformation or transduction. It cannot as yet be ruled out, however, that the appearance of CD cells may have been overlooked because of the rapid turnover occurring during a transformation process. Judging by results with the presently employed technique, there is not a great deal to indicate that a transformation may have taken place at the chromosomal level. The proliferation of the injected bone marrow in the irradiated homologous host indicates cellular repopulation as a possible means of recovery from irradiation. This is of extreme interest to us, and it has a direct bearing on our work.

In our investigations, the injection of mouse embryos with either a homologous or heterologous tumor transplant results in the growth of a tumor that makes its appearance sometimes as late as 30 days *post partum*. The characteristics of the original tumor, checked by transplantation back into the original host, are retained as long as tumor cells are transplanted serially through the embryos of the homologous host or through the brain tissue of newborn mice. Once adaptation to an adult of a previously unsusceptible species is accomplished—and this may take place, depending on the type of tumor, after 1, 4, or 8 passages through embryos—the characteristics of the derivative tumor line undergo striking changes. This is not the place to enumerate these alterations, but it is important to stress that, in spite of the changes, some of the prototype characteristics are never lost, since the tumor may be returned to its original host, where it grows progressively and invasively. These observations that the tumor homotransplants or heterotransplants "repopulate" the new "tolerant" host *post partum* are in line with the findings of Lindsley.

In contrast to the hypothesis that the injected homologous bone-marrow cells may elaborate some humoral substance that stimulates the recovery of the indigenous marrow, it is quite feasible that, in the course of repeated embryonic passages, the host tissue may produce a factor that alters the growing tumor cells without being able to deprive them of their identity. The possibility of transduction cannot be excluded.

The phenomenon of tolerance seems to be related strictly to cellular elements. Attempts to produce "tolerance" by either of the presently available methods through subcellular elements such as, for instance, virus, have so far usually ended in failure. One exception is the Rous sarcoma virus, which is so intimately associated with its tumor host cell.³ It is hard to say whether there is any other exception, but it might be worthwhile to study acquired tolerance

as a possible mechanism in the production of leukemia and salivary-gland tumors by filtrates of AKR mouse tissue.^{4, 5} The whole subject offers many opportunities for further investigation.

References

1. LORENZ, E., C. CONGDON & D. UPHOFF. 1952. Radiology. **58**: 863.
2. MAIN, J. M. & R. T. PREHN. 1955. J. Natl. Cancer Inst. **15**: 1023.
3. SIMONSEN, M. 1955. Nature. **175**: 763.
4. GROSS, L. 1953. Proc. Soc. Exptl. Biol. Med. **83**: 414.
5. STEWART, S. E. 1955. J. Natl. Cancer Inst. **16**: 41.

Part II. Cellular Antibodies, Serum Antibodies, and Delayed Hypersensitivity Phenomena

SIMILARITIES BETWEEN HOMOGRAFT REJECTION AND TUBERCULIN-TYPE ALLERGY: A REVIEW OF RECENT EXPERIMENTAL FINDINGS*

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The classical concepts of bacterial allergy of the delayed type are currently undergoing continuous change.¹⁻⁴ It would therefore be an imprudent oversimplification to expect to draw any very clear-cut analogies between the mechanism of the tuberculin type of allergic inflammatory response and that of an infinitely more complex biological phenomenon, homograft rejection. An examination of the 2 superficially unrelated phenomena, however, does suggest a basic similarity in behavior, in relation to data obtained in both systems with such techniques as are presently available.

Of the varieties of hypersensitive states that are probably operative via an immune response, bacterial allergy of the delayed tuberculin type occupies an unsettled position. This stems largely from the lack of an exact knowledge of the mechanism underlying its induction and development. The resolution of this dilemma is retarded by the fact that neither the antigen that incites delayed hypersensitivity, nor the antibody that mediates it, have been isolated. In nature, delayed bacterial allergy follows upon infection with diverse microorganisms, including bacteria, viruses, fungi, and spirochetes. It may also be acquired following contact with such noninfectious materials as plant products and simple chemical compounds.

In certain bacterial infections the pathogenesis of lesions, the symptomatology, and the course of the disease are host responses that may be conditioned by the presence of delayed hypersensitivity to products of the specific *Bacterium*. A classic example of this occurs in tuberculous infection with its characteristic accompaniments: an indolent course, granulomatous lesions, and a preferential intracellular residence of the microbe.

A postulated requirement for the induction of bacterial allergy is the interaction between intact bacterial cells and the tissues of the host in inflammatory lesions.⁵⁻⁷ The injection of extracted components of bacterial cells in soluble form usually results in the production of an Arthus type of sensitivity.^{8,9} That this may not always be the case has been shown by the observations of Dienes,¹⁰ of Freund,¹¹ and of Raffle¹² on the one hand, and of Mote and Jones,¹³ Gell and Hinde,¹⁴ Tremaine and Jeter,¹⁵ and Metaxas¹⁶ on the other. A new experimental approach that offers an additional exception to the need for intact bacterial cells is the production of delayed allergy by specific antigen-

* This work was conducted under the sponsorship of the Commission on Streptococcal Diseases, Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General, Department of the Army, Washington, D. C., and in part by the Atomic Energy Commission, Washington, D. C.

antibody precipitates reported by Uhr, Salvin, and Pappenheimer in this monograph.¹⁷

The development of sensitivity of the delayed type usually occurs within 10 to 14 days after infection or contact with the sensitizing material, although it may occur earlier. This latent period agrees well with that required for the formation of conventional antibody to known antigens. When sensitization has been induced, its presence can be detected by an intradermal test with antigens prepared from the particular causative agent.

In the absence of a measurable antigen-antibody system, the most compelling evidence linking bacterial allergy of the delayed type with an immune mechanism is the need for prior sensitization and the high degree of specificity exhibited in such sensitized states. Advantage is taken of this fact in clinical medicine each time the tuberculin, histoplasmin, or brucellergin skin test is performed.

There has been some confusion regarding the designation of this type of hypersensitivity as "delayed." In this instance the term is used to describe the usual lag period of 24 to 48 hours between the performance of the skin test in the sensitized subject and the subsequent development of maximal macroscopic evidences of the sensitized state. It does not mean, however, that all hypersensitive responses that are delayed in time are necessarily of the tuberculin type. The Arthus response, although classically an immediate type of hypersensitivity, can follow the same temporal sequence as the tuberculin reaction and be indistinguishable from the latter macroscopically. In such instances, only the histological nature of the lesion and serum and cell transfer studies can effectively dissociate the 2 responses. If both types of response are present simultaneously, as frequently may be the case, their dissociation becomes a more complicated issue.

In hypersensitive states of the delayed tuberculin type, the degree of sensitivity exhibited by the tissues appears to have no relation to measurable serum antibody.^{18, 19, 20} Moreover, such sera are not usually effective in the transfer of delayed sensitivity. Exceptions to this observation are the reports of Zinnser and Mueller²¹ and, more recently, of Cole and Favour.²² The transfer of delayed sensitivity by means of cells of the leukocyte series, however, has been consistently effective in animals^{23, 24} and in man.^{25, 26}

In the delineation of delayed sensitivity there has been a good deal of *in vitro* evidence collected to demonstrate a specific cytotoxic effect of antigen upon explanted cells obtained from sensitive subjects.^{27, 28} Such observations have been confirmed²⁹⁻³¹ or not confirmed^{32, 33} with almost equal frequency. This problem is reviewed by Favour³⁴ elsewhere in these pages.

Although the nature of *in vitro* cytotoxicity of antigen and its relationship to the delayed allergic inflammatory response *in vivo* are largely unsettled problems, the phenomenon is cited as one of the cardinal differences between the delayed and other types of hypersensitive response.^{1, 3, 4} Evidence for a direct cytotoxic effect of antigen *in vivo*, without the mediation of blood vessels or smooth muscle, has accrued from the consistent occurrence of the delayed response in the avascular cornea of sensitized animals.³⁵

The properties peculiar to delayed hypersensitivity enumerated above derive much of their significance from contrast with those exhibited by the wheal and erythema and the Arthus types of hypersensitive responses. The latter types of hypersensitive response are classified under the heading of "early responses" by Chase¹ to indicate the accelerated appearance of grossly visible effects that follow the application of test antigen. The time lag in this instance is usually a matter of minutes, although exceptions may occur as indicated above. In addition to the accelerated temporal sequence of events, the "early responses" share in common the presence of a measurable serum antibody, the titer of which parallels the degree of sensitivity in the host and governs the degree of sensitivity achieved following passive transfer by such sera to nonsensitive subjects. This property has been quantitated for Arthus sensitivity by Benacerraf and Kabat.³⁶ The dependence of "early responses" upon the presence of blood vessels or smooth muscle for their manifestations is again suggested by the failure of such reactions to occur in the avascular cornea³⁵ and the characteristic absence of *in vitro* cytotoxicity when specific antigen bathes sensitive tissues in culture.³⁷

The similarities and differences between the early and delayed types of hypersensitive responses are summarized in TABLE 1. For the sake of simplicity, in this table, emphasis has been placed on the differences between the 2 categories of hypersensitive response. It should be stressed, however, that such a table, although useful, is sometimes misleading, and perhaps represents an artificial separation based upon the touchstone of measurable serum antibody. For example, it is possible that there exists in the economy of nature only one type of hypersensitive response. Whether a particular hypersensitive

TABLE 1*
TYPES OF ALLERGIC INFLAMMATORY RESPONSES

	Early responses		Delayed responses
	Wheal and erythema	Arthus or anaphylactic	
Clinical state	Hay fever, asthma	Serum sickness	Tuberculosis, lymphogranuloma, histoplasmosis, syphilis, poison ivy
Sensitizing material	Pollens	Soluble proteins, carbohydrates	Bacteria, viruses, fungi, spirochetes, plant materials, simple chemicals
Antibody	Present in serum Nonprecipitable	Present in serum Precipitable	Absent in serum
Transfer of sensitivity	Heat labile With serum	Heat stable With serum	Absent in cells Unknown
Cytotoxicity of antigen for explanted sensitive cells	None	None	Not with serum With cells Present

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state appears in connection with antibodies that have or have not been shown to precipitate in the presence of antigen, or in connection with no measurable antibody (that is, not detectable within the limits of sensitivity of existing techniques), may be a quantitative expression of its intensity rather than an actual qualitative difference. This premise was intimated by the results of earlier observations^{18, 38} and has been supported in more direct fashion by the result of more recent experimental approaches to this problem.¹⁴⁻¹⁷

The delayed type of allergic inflammatory response appears as a recurrent general biological theme underlying the mechanism of the host's reactions that result in a wide variety of abnormal tissue states. It has been implicated in the development of allergic encephalomyelitis,^{39, 40} allergic neuritis,⁴¹ uveitis,⁴² aspermatogenesis,⁴³ and the Shwartzman reaction.⁴⁴

A probable manifestation of this widespread biological response, of particular concern to this monograph, is the rejection phenomenon evoked by transplanted normal tissues and by tumor tissues. The concept that the homograft-rejection phenomenon is mediated by the host's acquisition of an immune response to the antigens of genetically unrelated tissues has received experimental support from the observations of Medawar.⁴⁵⁻⁵¹

Medawar found that initial skin grafts applied to animals are rejected in about 10 days, a time consistent with that required to develop antibody and to develop delayed sensitivity. The rejection of a second graft from the same donor is accelerated (4 to 6 days). The latter reaction is a vigorous inflammatory response, and is a highly specific remembrance of prior sensitization. The dosage of tissue used to sensitize the recipient conditions the tempo and intensity of the rejection process. The mechanism of accelerated rejection cannot be transferred to nonsensitive animals by means of serum obtained from specifically sensitized donors, and there has been no antibody found in serum to mediate this highly specific response. Moreover, tissue-culture explants of donor skin are not affected by the presence of sera, cells, or cell extracts obtained from animals sensitized to the donor's skin.

In addition, Medawar demonstrated that the rabbit could be sensitized to a donor's skin by a preparatory injection of leukocytes obtained from the same donor.⁴⁵ It is of interest that sensitization was most often successful if the leukocytes were injected intradermally rather than intravenously. A recent confirmation and extension of this finding, using epidermal cell suspensions, has been reported by Billingham and Sparrow,⁵² and is discussed by Billingham⁵³ in this publication.

That the immune response to homografts represents the acquisition of hypersensitivity of the delayed tuberculin type is suggested by the timing of events, the absence of measurable serum antibody, the failure to transfer the response with serum, and the requirement that sensitization be by means other than transplantation be via the intradermal as opposed to the intravenous route.

The similarities between the tuberculin-type hypersensitivity and homograft-rejection hypersensitivity are summarized in TABLE 2.

We have noted earlier the exceptions to the requirement of intact bacterial cells for the induction of the tuberculin-type sensitivity. It begins to appear that there may also be exceptions to the requirement for intact tissue cells to

TABLE 2
ANALOGIES BETWEEN TUBERCULIN-TYPE HYPERSENSITIVITY
AND HOMOGRAFT REJECTION

	Tuberculin-type hypersensitivity	Homograft rejection hypersensitivity
Induction of sensitivity	Intact bacterial cells	Intact tissue cells
Latent period	10-14 days	10-12 days
Result of challenge	Koch phenomenon	Accelerated rejection
Specificity	For specific bacterium	For specific donor
Presence of measurable serum antibody	Variable	Variable
Parallelism between degree sensitivity and serum antibody	Not demonstrated	Not demonstrated
Transfer of sensitivity with serum	Negative	Negative
Transfer of sensitivity with cells	Positive	Positive
Cytotoxicity of antigen for ex-planted cells	Present	None

induce homograft sensitivity. This is suggested by the finding of Billingham and Sparrow⁵² that sensitization to skin homografts follows the intradermal injection of cell-free supernatant solutions prepared by washing dissociated epidermal cells in citrate-saline. This property is abolished following millipore filtration of such supernatant solutions.

The sequence of events that follows the initial exposure of the host to a foreign tissue (first-set reaction) and its relationship to events that follow upon a second exposure to the same tissue at a later time (accelerated rejection) suggest analogies to the Koch phenomenon described in tuberculous infection.^{1, 3, 4} Here the normal guinea pig, upon initial exposure, exhibits little or no response to the subcutaneous injection of living tubercle bacilli until 10 to 14 days have elapsed. About this time a hard, localized nodule develops at the site of inoculation and progresses to necrosis and ulceration. Guinea pigs that have responded in this fashion and are reinoculated with living tubercle bacilli from 4 to 6 weeks later exhibit a prompt and vigorous accelerated inflammatory reaction at the site of inoculation within 24 to 48 hours. This indurated, hemorrhagic area rapidly undergoes necrosis, ulceration, and subsequent healing. The initial response of the nonsensitive animal represents the gradual acquisition of delayed hypersensitivity to the products of the tubercle bacillus; the subsequent responses of the animal, when challenged, represent manifestations of this highly specific sensitized state.

More direct evidence in favor of the similarity between the homograft-rejection phenomenon and the delayed tuberculin type of hypersensitivity has been presented recently with the transfer of the accelerated rejection phenomenon by means of lymph node cells. Billingham *et al.*⁵³ demonstrated that a specific strain of mice (A) injected *in utero* with blood or tissue mash from another specific strain (B) will subsequently tolerate a skin homograft from the latter (B) for prolonged periods. Acquired tolerance induced in this fashion was abolished following the transplantation of lymph nodes obtained from normal

mice of the host strain (A). It is of interest that lymph nodes obtained from animals sensitized to the skin carried by the tolerant animal conferred upon the latter the capacity to reject the homograft in an accelerated fashion (that is, in the manner of one actively sensitized). It should be noted, however, that although lymph nodes obtained from nonsensitive animals also conferred the capacity to reject the homograft, it was at a much slower rate.

Billingham, Brent, and Medawar have recently^{55, 56} transferred the phenomenon of accelerated homograft rejection to normal nonsensitive mice by means of intraperitoneal inoculation of cells obtained from regional lymph nodes or spleen of specifically sensitized donors. Nonviable tissues, whole blood, serum, and leukocyte concentrates were ineffective for this purpose. Similar results had previously been obtained when tumor-tissue sensitivity was conferred on normal mice by Mitchison^{57, 58} following the intraperitoneal injection of lymph-node cells obtained from sensitized mice.

In addition, Mitchison and Dube⁵⁹ demonstrated the presence of a hemagglutinating antibody in the donor's serum, cytotoxic for the cells of the tumor, that was transferred to recipients by the same cells that mediated the accelerated rejection of the tumor. Because the antibody appeared after breakdown of the sensitizing graft in the donor and subsequent to the induced accelerated rejection of the tumor in the recipient following cell transfer, it was concluded that the hemagglutinating antibody is distinct from the antibody mediating accelerated homograft rejection. This conclusion is consistent with the data obtained, although it is equally probable that homograft rejection could be mediated by the hemagglutinating antibody, with the latter escaping detection at the time of tissue destruction because of the great differences in the relative sensitivities of the biological as compared to the serological test. It is pertinent here to recall Chase's¹ finding that the type of sensitivity conferred upon the recipient following cell transfer is dependent upon the mode of sensitization of the donor. Chase has shown that delayed dermal sensitivity and serum antibody to the same test material can be transferred to the recipient simultaneously by means of cells obtained from donors deliberately sensitized for this purpose.

Additional results supporting the similarity to bacterial allergy have been obtained by Algire and his colleagues,^{60, 61} employing a different technique, and these results are reported in this monograph.⁶² Transplanted normal or tumor tissues can survive indefinitely in diffusion chambers impermeable to recipient leukocytes and macrophages, but allowing free diffusion of extracellular fluids. Destruction of the transplant occurred if the diffusion chamber was permeable to the sensitive recipient's leukocytes or if washed splenic cells from specifically sensitized mice were added to the cell-impermeable chamber.

It is pertinent to consider briefly at this point the role of serum antibodies in the mediation of homograft rejection. There is a substantial volume of experimental evidence to indicate that serum antibodies are formed in response to the antigens of homografted tissues. Such antibodies have not been shown to precipitate in the presence of antigen, and they are usually demonstrated by hemagglutination techniques or by cytotoxicity studies.⁶³⁻⁶⁹ The demonstration of a serum antibody with specific cytotoxic properties deserves serious

consideration in any inquiry into the mechanism of homograft rejection, particularly since inhibition of the development of tissues exposed to immune sera becomes manifest upon subsequent transplantation.^{63, 66-69}

The findings in agammaglobulinemic patients presented by Good and by Porter in this monograph perhaps offer further support for the role of serum antibody in the mediation of homograft rejection. Good and Varco⁷⁰⁻⁷² demonstrated that an agammaglobulinemic patient does not reject a skin homograft, and Porter⁷³ and Good⁷² have shown that the agammaglobulinemic patient can develop delayed allergy to tuberculin and to 2,4-dinitrofluorobenzene. The extrapolation of data obtained in separate studies of a syndrome that must still be fully defined can be misleading, but it is particularly pertinent to note the development of delayed hypersensitivity of the tuberculin type despite homograft tolerance. This would suggest that the agammaglobulinemic individual lacks the capacity to recognize and react against tissues that are foreign to him—a deficiency also reflected in a parallel inability to form detectable serum antibody. It is not known whether the presence of serum antibody is necessary for the mediation of homograft rejection or whether this is merely another manifestation of a similar deficiency of mechanism. In any event, it would appear that the mechanism underlying the homograft-rejection phenomenon requires something in addition to that utilized in the development of delayed hypersensitivity by the agammaglobulinemic individual.

Thus the choice between the Arthus type and the delayed tuberculin type of hypersensitivity to explain the immunological mechanism underlying the homograft rejection phenomenon cannot be made with any degree of assurance. The 2 interpretations, however, need not contradict each other, since it is becoming increasingly probable that the 2 types of hypersensitivity may represent variations of a similar, if not the same, immunological mechanism. This is suggested by the data of Gell and Hinde,¹⁴ which are interpreted to indicate that delayed hypersensitivity is an early and incomplete phase of Arthus sensitization. Moreover, Uhr, Salvin, and Pappenheimer¹⁷ have presented direct evidence in support of the role of serum antibody in the induction of delayed hypersensitivity.

In closing, it is appropriate to recall the difficulties that arose in relation to the interpretation of the tuberculin reaction at the beginning of this century.⁷⁴ Clarification followed the demonstration that both the polysaccharide and the protein fractions of the tubercle bacillus may induce serum antibody formation and anaphylactic sensitivity that may parallel, yet be unrelated to, the state of delayed sensitivity of the tissues.^{5, 18, 19, 21, 75-78} The capacity to elicit the delayed allergic inflammatory response in tuberculin-sensitive subjects, however, was ultimately shown to be a property of the protein fraction of the tubercle bacillus.^{8, 9}

The possibilities for confusion in relation to the myriad of antigens possessed by skin and other tissues suggest that any definitive interpretation of the mechanism of homograft rejection would be helped immeasurably by a precise knowledge of the antigen or antigens against which it is directed and of the antibody or antibodies which mediate it.

References

1. CHASE, M. W. 1952. The allergic state. *In* Bacterial and Mycotic Infections of Man. R. J. Dubos, Ed. 2nd ed. **1**: 198. J. B. Lippincott. Philadelphia, Pa.
2. LAWRENCE, H. S. 1956. The delayed type of allergic inflammatory response. *Am. J. Med.* **20**: 428.
3. RAFFEL, S. 1953. Immunity, Hypersensitivity, Serology. : 245. Appleton-Century-Crofts, Inc. New York, N. Y.
4. WILSON, G. S. & A. A. MILES. 1955. Topley and Wilson's Principles of Bacteriology and Immunity. 4th ed. **2**: 1297. Williams & Wilkins. Baltimore, Md.
5. PETROFF, S. A. & F. W. STEWART. 1925. Immunological studies in tuberculosis. III. Concerning allergic reactions obtained in animals sensitized with killed tubercle bacilli. *J. Immunol.* **10**: 677.
6. DERICK, C. L. & H. F. SWIFT. 1929. Reactions of rabbits to non hemolytic streptococci. I. General tuberculin-like hypersensitivity, allergy or hyperergy following the secondary reaction. *J. Exptl. Med.* **49**: 615.
7. SWIFT, H. F. & C. L. DERICK. 1929. Reactions of rabbits to non-hemolytic streptococci. II. Skin Reactions in intravenously immunized animals. *J. Exptl. Med.* **49**: 883.
8. RICH, A. R. 1941. The significance of hypersensitivity in infections. *Physiol. Revs.* **21**: 70.
9. RICH, A. R. 1951. Hypersensitivity. *In* The Pathogenesis of Tuberculosis. 2nd ed. : 1, 388. C. C. Thomas. Springfield, Ill.
10. DIENES, L. 1929. The technique of producing tuberculin type of sensitization with egg white in tuberculous guinea pigs. *J. Immunol.* **17**: 531.
11. FREUND, J. 1955. The mode of action of immunologic adjuvants. *In* Advances in Tuberculosis Research. **7**. S. Karger. Basel, Switzerland.
12. RAFFEL, S. 1950. Chemical factors involved in the induction of infectious allergy. *Experientia*. **6**: 410.
13. MOTE, J. R. & T. D. JONES. 1936. The development of foreign protein sensitization in human beings. *J. Immunol.* **30**: 149.
14. GELL, P. G. H. & I. T. HINDE. 1954. Observations on the histology of the Arthus reaction and its relation to other known types of skin hypersensitivity. *Intern. Arch. Allergy and Appl. Immunol.* **5**: 23.
15. TREMAINE, M. M. & W. S. JETER. 1954. Passive cellular transfer of hypersensitivity to serum antigens in rabbits. *J. Immunol.* **74**: 96.
16. METAXAS, M. N. & M. METAXAS-BÜHLER. 1954. Frühreaktion und Spätreaktion bei der Serumallergie des Meerschweinchens und ihre Trennung durch passive Übertragung. *Allgem. Pathol. und Bakteriologie*. **17**: 128.
17. UHR, J. W., S. B. SALVIN & A. M. PAPPENHEIMER, JR. 1957. Production of delayed sensitivity to protein without detectable circulating antibody. *Ann. N. Y. Acad. Sci.* **64**(5): 877.
18. FREUND, J. & E. L. OPIE. 1937. An experimental study of protective inoculation with heat-killed tubercle bacilli. *J. Exptl. Med.* **66**: 761.
19. FOLLIS, R. H., JR. 1938. The effect of preventing the development of hypersensitivity in experimental tuberculosis. *Bull. Johns Hopkins Hospital*. **63**: 283.
20. ANGEVINE, D. M. 1941. A comparison of cutaneous sensitization and antibody formation in rabbits immunized by intravenous or intradermal injections of indifferent or hemolytic streptococci and pneumococci. *J. Exptl. Med.* **73**: 57.
21. ZINSSER, H. & J. H. MUELLER. 1925. On the nature of bacterial allergies. *J. Exptl. Med.* **41**: 159.
22. COLE, L. R. & C. B. FAVOUR. 1955. Correlations between plasma protein fractions, antibody titers, and the passive transfer of delayed and immediate cutaneous reactivity to tuberculin PPD and tuberculopolysaccharides. *J. Exptl. Med.* **101**: 391.
23. CHASE, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc. Soc. Exptl. Biol. Med.* **59**: 134.
24. CHASE, M. W. 1953. Immunological reactions mediated through cells. *In* The Nature and Significance of the Antibody Response. : 156. A. M. Pappenheimer, Jr., Ed. Columbia Univ. Press. New York, N. Y.
25. LAWRENCE, H. S. 1949. The cellular transfer of cutaneous hypersensitivity to tuberculin in man. *Proc. Soc. Exptl. Biol. Med.* **71**: 516.
26. LAWRENCE, H. S. 1955. The transfer in humans of delayed skin sensitivity to streptococcal M substance and to tuberculin with disrupted leucocytes. *J. Clin. Invest.* **34**: 219.

27. ARONSON, J. D. 1931. The specific cytotoxic action of tuberculin in tissue culture. *J. Exptl. Med.* **54**: 387.
28. RICH, A. R. & M. R. LEWIS. 1932. The nature of allergy in tuberculosis as revealed by tissue culture studies. *Bull. Johns Hopkins Hospital.* **50**: 115.
29. MOEN, J. K. & H. F. SWIFT. 1936. Tissue culture studies on bacterial hypersensitivity. I. Tuberculin sensitive tissues. *J. Exptl. Med.* **64**: 339.
30. HEILMANN, D. H., W. H. FELDMAN & P. C. MANN. 1944. Specific cytotoxic action of tuberculin: quantitative studies in tissue cultures. *Am. Rev. Tuberc.* **50**: 344.
31. GANGAROSA, E. J., J. T. INGLEFIELD, C. G. A. THOMAS & H. R. MORGAN. 1955. Studies on hypersensitivity of human tissues *in vitro*. I. Tuberculin hypersensitivity. *J. Exptl. Med.* **102**: 425.
32. BALDRIDGE, G. D. & A. M. KLIGMAN. 1951. Nature of tuberculin reaction: failure to demonstrate *in vitro* cytotoxicity for cells of sensitized animals. *Am. Rev. Tuberc.* **63**: 674.
33. MARKS, J. & D. M. JAMES. 1953. The effect of tuberculin on sensitized and normal leucocytes. *J. Hyg.* **51**: 340.
34. FAVOUR, C. B. 1957. *In vitro* studies on cell injury in the tuberculin type reaction: implications in homotransplantation. *Ann. N. Y. Acad. Sci.* **64**(5): 842.
35. RICH, A. R. & R. H. FOLLIS, JR. 1940. Studies on the site of sensitivity in the Arthus phenomenon. *Bull. Johns Hopkins Hospital.* **66**: 106.
36. BENACERRAF, B. & E. A. KABAT. 1950. Quantitative study of the Arthus phenomenon induced passively in the guinea pig. *J. Immunol.* **64**: 1.
37. ARONSON, J. D. 1924. Tissue culture studies on the relation of the tuberculin reaction to anaphylaxis and the Arthus phenomenon. *J. Immunol.* **9**: 259.
38. DIENES, L. & T. B. MALLORY. 1932. Histological studies of hypersensitive reactions. *Am. J. Pathol.* **8**: 689.
39. LUMSDEN, C. E., E. A. KABAT, A. WOLF & A. E. BEZER. 1950. Studies on acute disseminated encephalomyelitis produced experimentally in rhesus monkeys. V. Complement-fixing antibodies. *J. Exptl. Med.* **92**: 253.
40. LIPTON, M. M. & J. FREUND. 1953. The transfer of experimental allergic encephalomyelitis in the rat by means of parabiosis. *J. Immunol.* **71**: 380.
41. WAKSMAN, B. H. & R. D. ADAMS. 1955. Allergic neuritis: an experimental disease of rabbits induced by the injection of peripheral nervous tissue and adjuvants. *J. Exptl. Med.* **102**: 213.
42. COLLINS, R. C. 1953. Further experimental studies on sympathetic ophthalmia. *Am. J. Ophthalmol.* **36**: 150.
43. FREUND, J., G. E. THOMPSON & M. M. LIPTON. 1955. Aspermatogenesis, anaphylaxis and cutaneous sensitization induced in the guinea pig by homologous testicular extract. *J. Exptl. Med.* **101**: 591.
44. STETSON, C. A. 1955. Studies on the mechanism of the Schwartzman phenomenon. *J. Exptl. Med.* **101**: 421.
45. MEDAWAR, P. B. 1944. The behaviour and fate of skin autografts and skin homografts in rabbits. *J. Anat.* **78**: 176.
46. MEDAWAR, P. B. 1945. A second study of the behaviour and fate of skin homografts in rabbits. *J. Anat.* **79**: 157.
47. MEDAWAR, P. B. 1946. Immunity to homologous grafted skin. I. The suppression of cell division in grafts transplanted to immunized animals. *Brit. J. Exptl. Pathol.* **27**: 9.
48. MEDAWAR, P. B. 1946. Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. *Brit. J. Exptl. Pathol.* **27**: 15.
49. MEDAWAR, P. B. 1948. Immunity to homologous grafted skin. III. The fate of skin homografts transplanted to the brain, to subcutaneous tissue, and to the anterior chamber of the eye. *Brit. J. Exptl. Pathol.* **29**: 58.
50. MEDAWAR, P. B. 1948. Tests by tissue culture methods on the nature of immunity to transplanted skin. *Quart. J. Microscop. Sci.* **83**: 239.
51. MEDAWAR, P. B. 1954. General problems in immunity. *In* Preservation and Transplantation of Normal Tissues. Ciba Foundation Symposium. G. E. W. Wolstenholme & M. P. Cameron, Ed. : 1. Little, Brown & Co. New York, N. Y.
52. BILLINGHAM, R. E. & E. M. SPARROW. 1955. The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homografts in rabbits. *J. Embryol. Exptl. Morphol.* **3**: 265.
53. BILLINGHAM, R. E. 1957. Studies on epidermal cell suspensions, with particular reference to problems of transplantation immunity. *Ann. N. Y. Acad. Sci.* **64**(5): 799.
54. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1955. Acquired tolerance of skin homografts. *Ann. N. Y. Acad. Sci.* **59**(3): 409.

55. BILLINGHAM, R. E., L. BRENT, P. B. MEDAWAR & E. M. SPARROW. 1954. Quantitative studies on tissue transplantation immunity. I. The survival times of skin homografts exchanged between members of different inbred strains of mice. *Proc. Roy. Soc.* **143**: 43.
56. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc. Roy. Soc.* **143**: 58.
57. MITCHISON, N. A. 1953. Passive transfer of transplantation immunity. *Nature*. **171**: 267.
58. MITCHISON, N. A. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J. Exptl. Med.* **102**: 157.
59. MITCHISON, N. A. & O. L. DUBE. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. II. The relation between hemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations. *J. Exptl. Med.* **102**: 179.
60. ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1954. Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in immunized mice. *J. Natl. Cancer Inst.* **15**: 493.
61. WEAVER, J. M., G. H. ALGIRE & R. T. PREHN. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. *J. Natl. Cancer Inst.* **15**: 1737.
62. ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1957. Studies on tissue homotransplantation in mice, using diffusion-chamber methods. *Ann. N. Y. Acad. Sci.* **64**(5): 1009.
63. HAUSCHKA, T. S. 1952. Immunologic aspects of cancer. *Cancer Research*. **12**: 615.
64. GORER, P. A. 1955. The antibody response to skin homografts in mice. *Ann. N. Y. Acad. Sci.* **59**(3): 365.
65. AMOS, D. B., P. A. GORER, B. M. MIKULSKA, R. E. BILLINGHAM & E. M. SPARROW. 1954. An antibody response to skin homografts in mice. *Brit. J. Exptl. Pathol.* **35**: 203.
66. AMOS, D. B. & E. D. DAY. 1957. Passive immunity against four mouse leukoses by means of isoimmune sera. *Ann. N. Y. Acad. Sci.* **64**(5): 851.
67. RAMBO, O. N., JR., R. B. FUSON & E. J. EICHWALD. 1957. Immune phenomena elicited by transplanted tumors. II. Cytotoxic effects of immune guinea pig sera on mouse cancer cells. *Ann. N. Y. Acad. Sci.* **64**(5): 994.
68. WOODRUFF, M. F. A. 1957. Cellular and humoral factors in the immunity to skin homografts: experiments with a porous membrane. *Ann. N. Y. Acad. Sci.* **64**(5): 1014.
69. BILLINGHAM, R. E. & E. M. SPARROW. 1954. Studies on the nature of immunity to homologous grafted skin, with special reference to the use of pure epidermal grafts. *J. Exptl. Biol.* **31**: 16.
70. GOOD, R. A. & R. L. VARCO. 1955. Successful homograft of skin in a child with agammaglobulinemia. *J. Am. Med. Assoc.* **159**: 713.
71. GOOD, R. A. & R. L. VARCO. 1955. A clinical and experimental study of agammaglobulinemia. *J. Lancet*. **75**: 245.
72. GOOD, R. A., R. L. VARCO, J. B. AUST & S. J. ZAK. 1957. Transplantation studies in patients with agammaglobulinemia. *Ann. N. Y. Acad. Sci.* **64**(5): 882.
73. PORTER, H. M. 1957. The demonstration of delayed type reactivity in congenital agammaglobulinemia. *Ann. N. Y. Acad. Sci.* **64**(5): 932.
74. BALDWIN, E. R. 1910. Studies in immunity to tuberculosis. I.(a) Hypersusceptibility or anaphylaxis. *J. Med. Research*. **22**: 189.
75. ZINSSER, H. 1921. Studies on the tuberculin reaction and on specific hypersensitivity in bacterial infection. *J. Exptl. Med.* **34**: 495.
76. LEWIS, P. A. & J. D. ARONSON. 1923. The standardization of tuberculin. *Am. Rev. Tuberc.* **7**: 404.
77. MUELLER, J. H. 1926. A chemical study of the specific elements of tuberculin. II. The preparation of a residue antigen from old tuberculin. *J. Exptl. Med.* **43**: 9.
78. ENDERS, J. F. 1929. Anaphylactic shock with partial antigen of the tubercle bacillus. *J. Exptl. Med.* **50**: 777.

OBSERVATIONS ON IMMUNOLOGICAL MANIFESTATIONS OF THE HOMOGRAFT REJECTION PHENOMENON IN MAN: THE RECALL FLARE*

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This investigation of homografts in man was undertaken to study the reactions to repeated skin homografts from the same donor and to evaluate the duration of the resulting sensitivity.

Immunological aspects of the rejection of skin homografts have been reported by Medawar (1954)¹ and Billingham, Brent, and Medawar (1954).² These investigators described the active sensitization pattern expressed by the host's reaction to skin transplants, and also the host's behavior when again placed in contact with an antigenic challenge from the same source. Second-set skin homografts have exhibited an accelerated form of rejection in the species studied (Medawar, 1944,³ 1945,⁴ 1946;⁵ Dempster, Lennox, and Boag, 1950;⁶ Lehrfeld, Taylor, and Converse, 1955;⁷ Dempster, 1952;⁸ Sparrow, 1953;⁹ Gibson and Medawar, 1942;¹⁰ Longmire and Smith, 1951;¹¹ and Baxter and Entin, 1951¹²). Reactions of the host to additional skin grafts from the same source have been described in rabbits, in rats, and in man (Billingham and Boswell, 1953;¹³ Lehrfeld, Taylor, and Converse, 1955⁷).

Data obtained in our laboratories previously (Rogers, Converse, Taylor, and Campbell¹⁸), support the results described in this report. The experimental method outlined below is similar to that used in previous studies. The results obtained in each series were also similar.

METHODS AND TECHNIQUES

A patient volunteer in the healing phase of fractures of the lower leg received skin homografts from two donor sources. Donor A supplied the first homograft. A second homograft was received 21 days later from donor B. The patient subsequently received skin homografts from the same donor B at 12-, 26-, 22-, and 80-day intervals (TABLE 1).

Techniques employed in this study and criteria for homograft rejection have been reported previously (Converse and Rapaport, 1956¹⁷).

Full-thickness grafts were applied to the volar aspect of the right and left forearms alternately. The criteria for homograft rejection, as previously reported, included development of erythema and edema around the graft, graft cyanosis and edema, absence of flow in the graft vessels, and capillary thrombosis. Development of escharification and sloughing confirmed the diagnosis of homograft rejection in each case.

*The work described in this paper was supported by a grant from the United States Atomic Energy Commission, Washington, D. C., and by a grant from the Milbank Memorial Fund, New York, N. Y.

TABLE 1

Source of homograft	Time interval (days)	Homograft-rejection period
Donor A	0	6-7
Donor B	21	9-10
Donor B	12	4
Donor B	26	4-5
Donor B	22	4-5
Donor B	80	9-10

OBSERVATIONS

Skin Homograft from Donor A

This graft survived until the sixth day. It was then surrounded by erythema and edema, with progressively increasing cyanosis and edema. Blood flow ceased on the seventh day. Vessel thrombosis and breakdown occurred on the eighth day. Escharification and sloughing followed within the next three days. The events leading to rejection occurred during the sixth and eighth postoperative days.

Skin Homografts from Donor B

(1) *First-set graft.* Events leading to the rejection of the graft were identical with those described previously for the rejection of skin homografts. They began on the ninth postoperative day and continued until the tenth day. At the time of rejection of the graft, the site on the contralateral arm, on which homograft A had been placed, had healed, was quiescent, and exhibited no change in appearance during the time of rejection of the first-set homograft from donor B.

(2) *Second-, third-, and fourth-set grafts.* Three additional skin homografts from donor B were applied to alternate sides of the forearms of the host. The second-set graft was applied 12 days after rejection of the first; the third-set was applied 26 days after rejection of the second; the fourth-set was applied 22 days after rejection of the third (see TABLE 1).

The time interval for rejection of the repeat homograft was accelerated in each instance, and each of the homografts from donor B applied after rejection of the first graft was the subject of an accelerated rejection and occurred within the fourth to fifth postoperative day in a manner characteristic of the second-set graft. Blood flow was observed in the second- and fourth-set grafts only. All the grafts exhibited a pale appearance at 24 hours. The graft vessels were filled with hemic elements at 48 hours and attained maximal dilatation on the third day. Thrombosis, capillary breakdown, and hemorrhage followed in rapid succession. Concomitant gross changes included intense surrounding erythema, edema, and graft cyanosis (FIGURE 1). Escharification was complete by the seventh day.

No significant differences were observed in the behavior of the second-, third-, or fourth-set grafts from donor B.

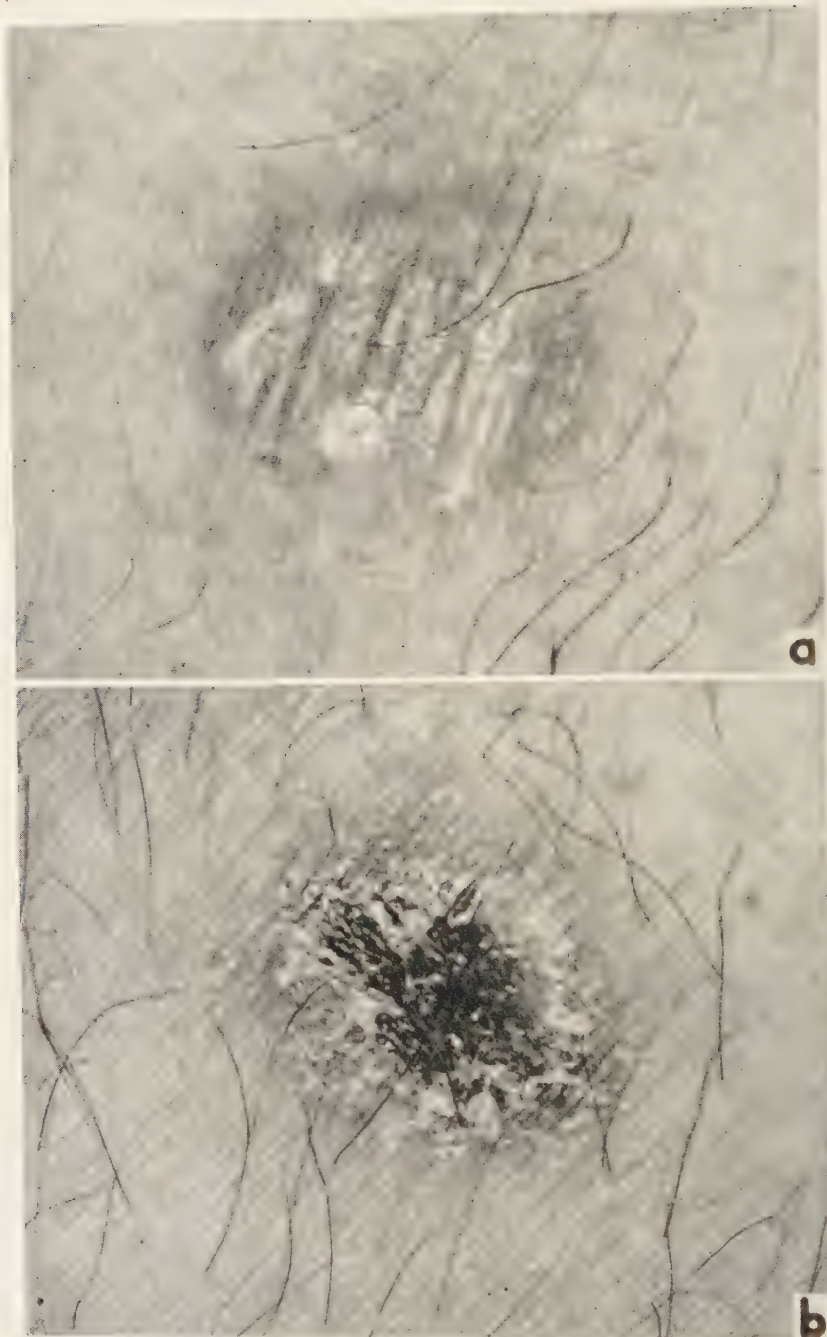


FIGURE 1. *A*, quiescent site of previously rejected skin homograft (homograft No. 3); *B*, recall flare and hemorrhagic necroses of previously quiescent skin-homograft site (homograft No. 3) at the time of rejection of homograft No. 4 from the same donor.

(3) *Fifth-set graft.* A fifth-set graft, applied 80 days after rejection of the fourth-set graft, was rejected by the ninth to tenth postoperative days.

The Recall Flare Phenomenon

The process of rejection of the second-, third-, and fourth-set grafts from donor B was associated with a unique phenomenon not previously reported in skin-transplantation studies.

The site of the preceding rejected homograft on the contralateral arm, up to the time of rejection of the second-, third-, and fourth-set graft, was quiescent and well-covered with host epithelium. At the height of the rejection reaction of each graft, however, this site exhibited a vigorous erythematous flare. In one instance, the flare progressed to intradermal hemorrhage and necrosis (FIGURE 1). In a number of cases, the flare was associated with intense pruritus.

The *recall flare* reached its maximal intensity within 1 day after the onset of the rejection changes of the subsequent graft, subsided within 48 hours, returned to a quiescent state, and was not observed again in each individual case.

The recall flare was not observed in the quiescent site of the rejected skin graft from donor A, or in the fourth-set graft site from donor B at the time of rejection of the fifth-set graft, which had again been rejected in the fashion of a first-set graft.

DISCUSSION

Observations in this study are in accord with previously reported manifestations of the second-set reaction. Challenge of a recipient with a skin homograft from one donor appears to result in the development of specific generalized sensitivity to the donor's skin. This is evidenced by the accelerated rejection of subsequent homografts from the same donor, but not from other donors. When this state of sensitivity has been attained, it is not enhanced further, in terms of a more rapid rejection. Subsequent skin grafts from the same donor are rejected in a fashion similar to that observed in the second-set homograft. This remains true during the entire period of the recipient's sensitivity to the donor's skin homografts.

The duration of sensitivity to skin homografts in other species has been described by Medawar (1944-1946),³⁻⁵ Billingham, Brent, and Medawar (1954),² and Lehrfeld, Taylor, and Converse (1955).⁷ The recipient in this study did not react in an accelerated fashion to the fifth-set homograft from donor B, but reverted to a first-set rejection pattern similar to that of the first-set graft from the same donor. At the time of maximal rejection of the fifth-set, the site of the fourth-set graft failed to show any recall-flare reaction. This fact suggests that the recipient's sensitivity to skin grafts from donor B was lost between the 26th and the 80th day after application of the last sensitizing graft.

Medawar (1954)¹ suggested a resemblance between the homograft-rejection phenomenon and the events leading to the development of hypersensitivity of the delayed bacterial type. Analysis of our data, particularly in terms of some of the manifestations of hypersensitivity of the delayed type, offers further evidence favoring this assumption.

Andrewes, Derick, and Swift (1926)¹⁴ noted a sequence of events after intradermal injection of nonhemolytic streptococci in rabbits consisting of a raised lesion with erythema that reached maximal size in 24 to 48 hours. This lesion regressed, showed a renewed increase in size and erythema at the eighth or ninth day, persisted for 2 to 3 days, and then receded. Andrewes and his associates designated these events as "primary" and "secondary reactions." After a second inoculation with the same microorganisms within 9 weeks of the first, a period of erythema began, as previously, and persisted for a longer period of time. The authors suggested that the primary and secondary reaction occurred simultaneously in this case, and that these reactions were probably superimposed. A subsequent attempt to relate this secondary reaction to the Arthus phenomenon was unsuccessful (Derick and Andrewes, 1926).¹⁵ These authors concluded that the secondary reaction was most probably analogous to the reaction observed in tuberculin sensitivity.

Lawrence (1949)¹⁶ studied the transfer of cutaneous sensitivity of the delayed type to tuberculin by means of peripheral blood leukocytes in man. He observed that some leukocyte recipients showed a flare at the site of previously negative tuberculin screening tests after the successful transfer of tuberculin sensitivity and related the appearance of the flare to the persistence of tuberculin at the test site.

The homograft-rejection phenomenon and its sequelae of accelerated rejection of subsequent grafts from the same donor appear analogous to the events observed by Andrewes, Derick, and Swift.

The development of the recall-flare reaction in our study parallels the events observed in sensitivity of the delayed type. The recall flare may be the result of the persistence at the rejected graft site of sufficient antigenic material to allow for the development of a new reaction when the individual is challenged at another site.

The recall flare failed to appear at the site of the fourth-set graft upon rejection of the fifth-set graft. At this time the individual reverted to a first-set pattern, suggesting that all antigen had disappeared from the fourth-set site, or that not enough antigen remained there to produce a visible reaction. This last observation suggests that specific sensitivity to skin homografts persists as long as the antigen or its derivatives are present in the host.

SUMMARY

(1) Skin homografts from two separate donors were applied to the same recipient at a 21-day interval. Both grafts behaved as first-set homografts.

(2) Repeated homografts from the same donor, placed on a recipient at 12-, 26-, and 22-day intervals, were rejected in the accelerated manner characteristic of second-set homografts.

(3) A skin homograft from this same donor, applied to the recipient 80 days after rejection of the fourth-set homograft, behaved as a first-set homograft.

(4) The accelerated rejection of each successive graft from the same donor was accompanied by a *recall flare* at the quiescent site of the preceding graft from the same donor. This flare subsided in 48 hours and was not observed

again in each individual case. This recall flare failed to appear when the recipient reverted to his original status, rejecting the fifth-set graft in a manner characteristic of the first-set skin homograft.

ACKNOWLEDGMENTS

The authors thank H. Sherwood Lawrence for his advice and Harry H. Shapiro for editorial assistance.

References

1. MEDAWAR, P. B. 1954. General problems of immunity. *In* Preservation and Transplantation of Normal Tissues. Ciba Foundation Symposium. : 1-22. London, England.
2. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1954B. *Proc. Roy. Soc.* **143**: 58.
3. MEDAWAR, P. B. 1944. *J. Anat.* **78**: 176.
4. MEDAWAR, P. B. 1945. *J. Anat.* **79**: 157.
5. MEDAWAR, P. B. 1946. *Brit. J. Exptl. Pathol.* **27**: 9.
6. DEMPSTER, W. J., B. LENNOX & J. BOAG. 1950. *Brit. J. Exptl. Pathol.* **31**: 670.
7. LEHRFELD, J. N., A. C. TAYLOR & J. M. CONVERSE. 1955. *Plast. Reconstr. Surg.* **15**: 74.
8. DEMPSTER, W. J. 1952-1953. *Brit. J. Plastic Surg.* **5**: 228.
9. SPARROW, E. M. 1953. *J. Endocrinol.* **9**: 101.
10. GIBSON, T. & P. B. MEDAWAR. 1942-1943. *J. Anat.* **77**: 299.
11. LONGMIRE, W. P., JR. & S. W. SMITH. 1951. *Arch. Surg.* **62**: 443.
12. BAXTER, H. & M. A. ENTIN. 1951. *Am. J. Surg.* **81**: 285.
13. BILLINGHAM, R. E. & T. BOSWELL. 1953B. *Proc. Roy. Soc.* **141**: 392.
14. ANDREWES, C. H., C. L. DERICK & H. F. SWIFT. 1926. *J. Exptl. Med.* **44**: 35.
15. DERICK, C. L. & C. H. ANDREWES. 1926. *J. Exptl. Med.* **44**: 55.
16. LAWRENCE, H. S. 1949. *Proc. Soc. Exptl. Biol. Med.* **71**: 516.
17. CONVERSE, J. M. & F. T. RAPAPORT. 1956. *Ann. Surg.* **143**: 306.
18. ROGERS, B. O., J. M. CONVERSE, A. C. TAYLOR & R. CAMPBELL. Unpublished data.

IN VITRO STUDIES ON CELL INJURY IN THE TUBERCULIN-TYPE REACTION: IMPLICATIONS IN HOMOTRANSPLANTATION

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Introduction

There are many provocative similarities between the tissue reaction that takes place at the site of a homotransplant rejection and those changes seen in the Koch phenomenon associated with cutaneous reinfectious tuberculosis. The purpose of this presentation is to review briefly some of the more important local events that occur during the tuberculin reaction. Particular attention is to be paid to those *in vitro* and semi-*in vitro* observations that have permitted a relatively isolated study of individual factors in tuberculin allergy. It is not clear that this artificial separation of its parts results in a valid explanation of the *in vivo* tissue responses to tuberculin. This schema will help, however, in presenting a number of recent observations of interest to students in the field of transplantation immunology.

Cellular Affinity

There is considerable evidence to show that the selective sites of cellular uptake of haptens or antigenic substance by tissues have a great deal to do with the patterns of subsequent allergic reactions that may follow. An examination of this feature in both immediate-type and delayed-type allergic responses is pertinent background information for interpreting the *in vitro* observations on tuberculin allergy to be described later.

In the case of immediate-type allergy, various tagging techniques, such as dye labeling¹ or fluorescent labeling of proteins² have been used to show that soluble substances that provoke an antibody response are distributed widely throughout the body. Characteristically, tagged antigens go to the walls of blood vessels, into cells of the spleen and lymph nodes, and into the developing plasma cells. Freund and Lipton³ have recently shown that antigens plus adjuvants also are distributed widely, even when the injection site is later excised from the host. It is presumed that the presence of plasma cells, generally believed to be the source of antibodies found in the γ -globulins of the plasma,⁴ indicates that an antigenic stimulus has taken place. On the contrary, when there is an absence of plasma cells (agammaglobulinemia), some types of antibody formation are impaired.⁵ In the light of these considerations it is not surprising that allergic reactions that do occur often are at the many sites of antigen deposit. In general, mild anaphylactic allergy is associated with smooth muscle spasm and capillary dilation, whereas more intense anaphylactic reactions are associated with capillary injury, visceral angitis, and the complex inflammatory pattern of the Arthus reaction.

Anaphylactic allergy also is characterized by the fact that many body structures are not involved. Among those that usually escape injury are the

peripheral blood leukocytes. This phenomenon also can be demonstrated *in vitro*. Aronson⁶ has shown that "tissue culture" explants of the buffy coat are not injured when they are exposed to horse serum and grown in high concentrations of antihorse serum. This is true even when the cells are derived from animals made strongly sensitive to this antigen.

Leukocytes are not always spared in anaphylactic-type cellular reactions, however. In fact, there are two types of antibody reactions that are directed toward antigens on or in leukocytes. The first of these was studied long ago by Bedson.⁷ Rabbits immunized with guinea pig leukocytes make an anti-leukocyte antibody that will cause leukopenia when given to guinea pigs. In recent years, attention has been called to an antileukocyte agglutinin found in the blood of patients with a variety of blood dyscrasias.^{8, 9} It is not clear that this antibody, which will agglutinate normal buffy coat white blood cells *in vitro*, actually does any harm to the patients' own white cells. Furthermore, careful studies by Rose Payne¹⁰ indicate that patients with this leukoagglutinin have almost invariably received 1, and usually many, transfusions. This antibody to leukocytes appears to develop in response to leukocyte antigens which are heterologous to the host.

The second kind of antibody that develops against white blood cells and can be detected *in vitro* is the type associated with granulocytopenia due to pyramidon.¹¹ Patients who show this complication of pyramidon therapy have in their blood an antibody that, by itself, is innocuous, but which will lyse both patient and normal leukocytes exposed to the specific drug. In this system the antibody is not directed toward the leukocyte unless it is exposed to the drug.

Another clear-cut example of allergic destruction of the target site is found in sedormid purpura. Ackroyd¹² has shown that an antibody that appears in the blood of the sensitized individual with purpura will injure the circulating platelets. Normal platelets from another person that have been exposed *in vitro* to the chemical also are promptly lysed by this antibody. Quinidine purpura is presumed to be mediated by a similar kind of antibody.¹³ In passing, it should be mentioned that purpura also can be produced with antiplatelet antibodies in a heterologous host in the same way that leukoagglutinins have been prepared.⁷

In these less common examples of cell injury of the anaphylactic type, circumstantial evidence suggests that the antigen or hapten against which the antibody is directed is affixed to the cell or is already a part of the cell which is injured at the time of antigen-antibody combination. In tuberculin allergy, a similar type of leukocyte injury *in vitro* has also been described. Certain circumstantial as well as direct observations suggest that cellular localization of tuberculin on the target cell is an important part of this reaction, just as it appears to be in anaphylactic allergy.

Among the indirect evidences of a cellular localization of tuberculin on cells injured in the tuberculin reaction are those obtained from skin-test studies on hosts before and after tuberculin sensitization. We have seen individuals who have been skin-tested and found to be negative reactors. Weeks to months later, at the time they then acquire tuberculin sensitivity, the initially negative

skin test sometimes reacts. This is some evidence that tuberculin must have remained affixed to one or another cell type in the skin during the latent interval.

Another kind of indirect evidence of tuberculin adsorption by tissues has been supplied by Pepys,¹⁴ who showed that histamine diminished and that adrenalin augmented tuberculin reactivity when those substances were given locally with a tuberculin skin test. It is thought that histamine hastens and that adrenalin delays diffusion of tuberculin from the test site.

More direct evidence that tuberculin is taken up by leukocytes, whether or not the host is tuberculin-sensitive, has been offered by Ritts and Favour,¹⁵ who showed that isotope-tagged tuberculin given intravenously to rabbits is taken up by peripheral blood leukocytes.

These various observations on direct cell injury indicate that, at the cellular level, both immediate- and delayed-type allergic responses may be mediated by a humoral factor in the blood. This factor can react either with a specific hapten or antigen that is an integral part of the cell, or it may cause cell injury by virtue of the cells' capacity to take up haptens or antigens to which the humoral factor is directed.

Toxic Effect of Tuberculin on Cells

One of the first investigators to study the direct toxic effect of tuberculin on cells was Holst,¹⁶ who used microhematocrit capillary tube preparations. Holst mixed fresh whole blood with tuberculin and watched the migration of leukocytes from the buffy coat. He also studied the toxic effect of tuberculin on cellular morphology and on the capacity of leukocytes to phagocytose non-specific bacteria. All 3 of these methods revealed a direct toxic effect of tuberculin on cells from the tuberculin-sensitive host. The role of possible plasma factors similar to those that occur in granulocytopenia was not evaluated.

Recently O'Neill and Favour¹⁷ have used a similar type of capillary-tube study to compare and contrast *in vivo* and *in vitro* tuberculin allergy in patients with pulmonary tuberculosis. They found that patients or normal subjects with a positive tuberculin test did not always have *in vitro* evidence of cellular sensitivity to tuberculin. Furthermore, they found that cell toxicity required very large amounts of tuberculin compared to the minuscule quantities active in the intact host. When they washed leukocytes and resuspended them in the plasma of compatible blood from other patients and from controls who had no tuberculin hypersensitivity, they found that much of the toxic effect of tuberculin could be attributed to factors present in the plasma. In some preparations, washed cells were sensitive to tuberculin even when suspended in normal plasma, but it could not be said that washing necessarily removed all the autologous plasma.

A much more publicized but less well analyzed *in vitro* system for observing the toxic effect of tuberculin on cells has been the tissue-culture method first used successfully in 1932 by Rich and Lewis.¹⁸ These workers presented example data showing that OT 1:60 was toxic for buffy coat explants cultured in autologous and, in some cases, in homologous plasma. A number of workers have used this method to demonstrate essentially the same findings. Moen

and Swift¹⁹ showed that the effect was disease specific as well as antigen specific. In this explant system and in the capillary tube method, very large amounts of tuberculin are necessary to produce results.²⁰ The polysaccharides and the nucleic acid from tubercle bacilli do not produce this effect.^{21,22} It should be emphasized that all of these successes with tissue cultures have employed cell preparations rich in lymphoid elements.

Not all workers who have used the tissue-culture method have achieved positive results. Long ago, Fischer,²³ the eminent tissue-culture worker, using 12th-passage explants of fibroblast from chicks infected with avian tubercle bacilli, found no toxic effect of avian tuberculin on tissue cultures. In the sense that a tissue culture is a cell colony adapted to its new environment that grows and multiplies, Fischer's work is unique. Other tissue-culture studies on tuberculin allergy have not used similarly controlled methods. Even with the modified type of culture, results are not always positive. A number of more recent workers have had negative results using a variety of *in vitro* methods,²⁴ including those quite similar to the ones described by Rich and Lewis. When cells have been derived from liver²⁵ and skin,²⁶ negative results have likewise followed. These negative *in vitro* results are not limited to cells of epithelial origin, for Warburg respiratory studies on polymorphonuclear leukocyte suspensions also have been negative.²⁷ In this connection it is interesting to note that predominantly neutrophilic cell suspensions derived from sensitized donors will not passively transfer tuberculin allergy²⁸ in the same manner as cell suspensions containing lymphocytes.²⁹ It would appear that, when tissue cultures have shown sensitivity to tuberculin, they have contained cells derived from the lymphoid series. On the other hand, as in capillary-tube methods, the presence of lymphoid cells does not insure tuberculin cytotoxicity. Neither is cutaneous reactivity to tuberculin necessarily regularly associated with *in vitro* tuberculin allergy in the cell populations isolated from the same host.

Another interesting *in vitro* method for studying the toxic effects of tuberculin on cells has been the cytolysis technique adapted to use in a number of laboratories. In this procedure, leukocytes derived from the peripheral blood,^{30, 31} the thoracic duct,³² the urine sediment,³³ the spinal fluid,³¹ or the spleen of the sensitized host³⁰ are incubated with tuberculin as suspensions, rather than as fixed cell masses. The index of cell toxicity is found from direct cell counts during incubation. Differential cell-counting permits some estimate of the number and types of cell injured by tuberculin in the first hour or more in which cell damage occurs. This system has been found to be antigen- and disease-specific.³⁰ It has been used to study rabbits,³¹ mice,³⁰ guinea pigs,³⁰ and humans.³⁵ Through its use, a number of features of the toxic effect of tuberculin on cells have been uncovered.

Various workers have noted that, in the cytolysis system, not all cells in suspensions are injured by tuberculin.^{30, 31, 36} Complement seems to be necessary for such cell lysis as does occur.³⁷ In animals such as mice, which do not exhibit a delayed type of tuberculin reaction to any appreciable degree,^{38, 39} the lymphocytes only are injured. When spleen cells are used, only the more mature lymphocytes are injured.³⁰ In the guinea pig and man, which exhibit

good delayed types of hypersensitivity, a proportion of both the segmented leukocytes and lymphocytes are injured. Another feature of the cytolysis system is the short time in which reactions occur. In any given cell population kept in motion by roller tubes, cytolysis reaches its maximum within 1 to 2 hours. On the other hand, Berdel⁴⁰ has found that cell injury by tuberculin in suspensions subject to occasional shaking shows critical differences as late as 54 hours, a time much more comparable to the situation present in buffy-coat tissue cultures. All workers with the cytolysis system have found subjects who have excellent cutaneous allergy, but not *in vitro* findings. As we have seen, much the same situation applies to other *in vitro* methods of studying the toxic effect of tuberculin.

A further analysis of the cytolysis system in guinea pigs sensitized to tuberculin indicates that washed preparations of peripheral blood lymphocytes will, on incubation, yield small amounts of a plasma factor.⁴¹ When this factor is used with tuberculin to suspend normal cells, some neutrophils are then lysed. Incubated neutrophils do not release such a plasma factor.

These *in vitro* observations in the mechanism of the tuberculin reaction emphasize the role played by lymphocytes in delayed-type allergic responses. Further attention has been called to this facet of delayed-type reactions by studies on patients with agammaglobulinemia. Individuals with this condition exhibit delayed-type bacterial allergy.⁴² Delayed-type allergy can be transferred to them by means of cells⁴³ and, as reported elsewhere in this monograph, they develop and maintain tuberculin reactivity following BCG vaccination.⁴⁴

At the present time our understanding of the tuberculin reaction on the cellular level embraces the following concepts:

- (1) Tuberculin first is taken up on selected cells of the host. In those capable of showing tuberculin reactivity, a key cell in this uptake is the segmented neutrophil. In both anergic and tuberculin reactive species it is likely that a portion of the mature lymphocytes also take up tuberculin. It is not clear from *in vitro* studies so far available whether other body-cell types possess a similar capacity to localize this biologically active substance. Although indirect evidence from skin testing suggests that one or more cell types in the skin also have this ability, this subject is not fully clarified.

- (2) Neutrophils that take up tuberculin are injured only if there is present in the blood a plasma factor that promotes this injury, or when they are intimately associated with lymphocytes from the sensitized host. Lymphocytes are injured by their own capacity to shed a plasma factor.

- (3) Under most circumstances, lymphocytes come into close cellular contact with the sites of cell injury. Postulated humoral factors derived from these cells, which effect the final injury to target cells, are most difficult to study. Observations on patients with agammaglobulinemia and on passive transfer of plasma components to these patients suggest that these elusive humoral factors do not belong to the usual class of antibodies found in the plasma gamma globulins.

- (4) Plasma factors associated with tuberculin allergy that have been found apparently are present only in transient and in small quantities in the circu-

lating blood. Furthermore, the studies with capillary tubes and those of Berdel with the 54-hour cytotoxicity test method suggest that, at times, the plasma may also contain a tuberculin-neutralizing substance that blocks the cytotoxic action of plasma factor by tuberculin. Some time ago Wells and Wylie⁴⁵ described a passively transferable property in the plasma of patients with tuberculin anergy due to sarcoidosis. More recently, Cole's and Favour's studies⁴⁶ with Fraction IV-10 and Fraction II of the plasma of the immunized guinea pig imply a similar competition of plasma components for one or more moieties on crude preparations of tuberculin now available for study.

The fragmentary nature of these various plasma studies is most interesting, but it is too nebulous to permit as clear an interpretation as those possible from *in vitro* observations with cells. Whatever the role of these plasma factors in tuberculin allergy may be, *in vitro* studies to date do not support the vague concept of a generalized tissue sensitivity dependent on mysterious properties persistent in all cells and independent of exposure to their humoral environment. Much the same conclusion can be drawn indirectly from the passive transfer studies of Chase.⁴⁷ Either by injection of cell suspensions⁴⁸ or by parabiosis,⁴⁹ passively transferred tuberculin allergy is quite transient once cell injections or parabiotic union are interrupted.

In view of these findings, the peripheral blood appears to be an unlikely place in which to find plasma factors responsible for delayed-type reactions. Furthermore, even the cell types that mediate tuberculin-type reactions may be present only in limited quantities in any given blood sample. Their best source is in the regional lymph nodes draining an antigen deposit.³ To a large extent the tuberculin-type reactivity created at other sites in the host, such as by tuberculin testing, may represent a passive transfer of reactivity within the host. Apparently, cellular and humoral components in the blood at any one time do not appear to be a direct measure of the progress of the local tuberculous infection that has sensitized the host.

Much the same statement can be made for any tuberculin-type reactivity which may be associated with tissue transplantation. We know that, under certain conditions, graft rejection can be accelerated by the passive transfer of cells from a sensitized donor.⁵⁰ When grafts are located in sites with a rich lymphatic system, the regional lymph nodes are a major source of internal "passive transfer" cells that eventually find their way back to the graft via the blood stream. When perigraft infiltrates can be avoided, as Glenn H. Algire has shown with his cell-impermeable membranes, heterologous tissue grafts may survive for long intervals. In those parts of the central nervous system where lymphatics are essentially absent, both grafts and tuberculous granuloma tend to be benign, encapsulated, space-occupying masses. This phenomenon calls to mind Landsteiner's and Chase's experiments⁵¹ in which they limited poison-ivy sensitization and an antibody response to horse serum by cutting through the lymphatics surrounding the injection sites. In contrast to brain substance, the rich lymphatic system of the skin is always ready to initiate a vigorous cellular reaction to foreign substances. Furthermore, as Hagermann⁵² has shown, fluorescent-tagged cells used to transfer passively drug sensitivity in animals are known to go to the skin.

We view this series of cellular phenomena seen in the tuberculin reaction as a very primitive host mechanism for sloughing off particulate foreign agents that elaborate sensitizing substances of a wide variety of types. From an immunological viewpoint, patients with agammaglobulinemia are like caterpillars. They have the elemental capacity to develop granulomas and to make cells of the lymphoid series. These mechanisms seem to possess some effectiveness in combating such intracellular parasites as viruses and tubercle bacilli. They are not a fortuitous kind of tissue response. Even when they result in a sacrifice of the whole tissue, as in the Koch phenomenon, they nevertheless constitute a means of ridding the host of a local noxious invader. On the other hand, they are ill-adapted for combating extracellular invaders such as homographs and pyogenic infections. In contrast, the more sophisticated immune mechanisms of anaphylaxis depending on plasma cells and their antibodies would seem to be a relatively modern animal acquisition. In immunological terms, this system has permitted a more certain survival of the individual but, in doing so, has made him more provincial with regard to foreign antigens in his intramural cellular milieu. These two "immune systems," variously interpreted, are the ones that form the basic immunologic dichotomy of immunity versus allergy that is taught in texts.

In some species, such as mice and rats, tuberculin-type reactivity is at a low level. It is interesting to observe that these animals reject homografts quite as readily as do species capable of showing tuberculin allergy. It is not clear whether or not this is the antithesis of successful homografting in agammaglobulinemia patients.

These considerations of murine anergy also prompt us to close these remarks by reemphasizing the large gaps in our knowledge concerning the allergic nature of transplant rejection. It will also be apparent that many fundamental enigmas still plague the serious student of delayed-type tuberculin allergy as well.

References

1. KRUSE, H. & P. D. McMASTER. 1949. The distribution and storage of blue antigenic azoproteins in the tissue of mice. *J. Exptl. Med.* **90**: 425.
2. COONS, A. H., H. J. CREECH, R. N. JONES & E. BERLINER. 1942. Demonstration of pneumococcal antigen in tissues by use of fluorescent antibody. *J. Immunol.* **45**: 159.
3. FREUND, J. & M. M. LIFTON. 1955. Experimental allergic encephalomyelitis after excision of the injection site of antigenadjuvant emulsion. *J. Immunol.* **75**: 454.
4. FAGRAEUS, A. 1948. Antibody production in relation to the development of plasma cells. Ulla Schott, Trans. *Esselte Akriealag*. Stockholm, Sweden.
5. BRUTON, O. C., L. APT, D. GITLIN & C. S. JANTWAY. 1953. Absence of serum gamma globulins. *Med. Ann. Dist. Columbia*. **32**: 648.
6. ARONSON, J. D. 1931. The specific cytotoxic action of tuberculin in tissue culture. *J. Exptl. Med.* **54**: 387.
7. BEDSON, S. P. 1921. Blood platelet antiserum, its specificity and role in the experimental production of purpura. *J. Pathol. Bacteriol.* **24**: 469.
8. GOUDSMITH, R., J. J. VAN LOCHENER, JR. 1953. Studies on the occurrence of leucocyte-antibodies. *Vox Sanguinis*. **3**: 3.
9. DANSSET, J., A. NENNA & H. BREGY. 1954. Leukoagglutinins in chronic idiopathic or symptomatic pancytopenia and paroxysmal nocturnal hemoglobinuria. *Blood*. **9**: 696.
10. PAYNE, R. Personal communication.
11. MOESCHLIN, S. & K. WAGNER. 1952. Agranulocytosis due to the occurrence of leucocyte-agglutinins. *Acta Haematol.* **8**: 29.

12. ACKROYD, J. F. 1949. The pathogenesis of thrombocytopenic purpura due to hypersensitivity to sedormid (allyl-isopropyl acetylcarbamide). *Clin. Sci.* **7**: 249.
13. LARSON, R. K. 1953. Mechanism of quinidine purpura. *Blood*. **8**: 16.
14. PEPPYS, J. 1953. Effect of local adrenalin and histamine on tuberculin reactions. *Acta Allergol.* **6**: 265.
15. RITTS, R. E., JR. & C. B. FAVOUR. 1955. *In vivo* uptake of isotope tagged tuberculin by leucocytes. *J. Immunol.* **75**: 209.
16. HOLST, P. M. 1921. Effects of tuberculin. *J. Hyg.* **20**: 342.
- 16a. HOLST, P. M. 1922. Studies on effects of tuberculin. *Tubercle*. **3**: 249, 289, 337.
17. O'NEILL, E. F. & C. B. FAVOUR. 1955. Tissue culture analysis of tuberculin hypersensitivity in man. *Am. Rev. Tuberc.* **72**: 577.
18. RICH, A. R. & M. R. LEWIS. 1932. The nature of allergy in tuberculosis as revealed by tissue culture studies. *Bull. Johns Hopkins Hospital*. **50**: 115, 134.
19. MOEN, J. K. & H. F. SWIFT. 1936. Tissue culture studies on bacterial hypersensitivity. *J. Exptl. Med.* **64**: 339.
20. FABRIZIO, A. M. 1952. Effect of purified fractions of tuberculin on leucocytes from normal and tuberculous animals in tissue culture. *Am. Rev. Tuberc.* **65**: 250.
21. HEILMANN, D. H. & P. B. SEIBERT. 1946. The effect of purified fractions of tuberculin on tuberculin-sensitive tissue. *Am. Rev. Tuberc.* **53**: 71.
22. HEILMANN, D. H., W. H. FEIDMAN & F. C. MANN. 1944. Specific cytotoxic action of tuberculin: quantitative studies on tissue culture. *Am. Rev. Tuberc.* **50**: 344.
23. FISCHER, A. 1928. Tuberkulin und Fibroplastin. *Z. Immunitätsforsch.* **56**: 24.
24. O'NEILL, E. F. & C. B. FAVOUR. *Op. cit.* Historical review section.
25. JACOB, F. & J. MARKS. 1953. On the tuberculin sensitivity of epithelial cells *in vitro*. *J. Hyg.* **51**: 541.
26. CRUICKSHANK, C. N. D. 1951. Sensitivity to tuberculin. *Nature*. **168**: 206.
27. MARKS, J. & D. M. JAMES. 1953. The effect of tuberculin on sensitized and normal leucocytes. *J. Hyg.* **51**: 340.
28. KIRCHHEIMER, W. F., A. E. HESS & R. G. SPEARS. 1951. Attempts at passive transfer of tuberculin-type of sensitivity with living granulocytes. *Am. Rev. Tuberc.* **64**: 516.
29. LAWRENCE, H. A. 1949. The cellular transfer of cutaneous hypersensitivity to tuberculin in man. *Proc. Soc. Exptl. Biol. Med.* **71**: 516.
30. FAVOUR, C. B. 1947. Lytic effect of bacterial products on lymphocytes of tuberculous animals. *Proc. Soc. Exptl. Biol. Med.* **65**: 269.
31. WAKSMAN, B. H. 1953. Studies of cellular lysis in tuberculin sensitivity. *Am. Rev. Tuberc.* **68**: 746.
32. WESSLEN, T. 1952. An histological study of the tuberculin reaction in animals with passively transferred hypersensitivity. *Acta Tuberc. Scand.* **26**: 175.
33. KANZLER, W. 1952. Beobachtung einer *in vitro* Zytolyse der Harnleukozyten bei der Tuberkulose der Harnorgane. *Z. Urol.* **45**: 569.
34. EWERBECK, H. & I. JÄGER. 1955. Die Tuberkulin-Empfindlichkeit der Leukocyten und Lymphocyten in Liquor cerebrospinalis als differentialdiagnostisches Symptom. *Tuberkulosearzt*. **9**: 348.
35. FAVOUR, C. B., P. FREMONT-SMITH & J. M. MILLER. 1949. Factors affecting the *in vitro* cytotoxicity of white blood cells by tuberculin. *Am. Rev. Tuberc.* **60**: 212.
36. CHAMBERLIN, R. E. 1952. Thesis. Univ. Michigan. Ann Arbor, Mich.
37. MILLER, J. M., J. R. VAUGHAN & C. B. FAVOUR. 1949. The role of complement in the lysis of leucocytes by tuberculoprotein. *Proc. Soc. Exptl. Biol. Med.* **71**: 592.
38. GERSTL, B. & R. M. THOMAS. 1940-1941. Experimental tuberculosis in mice. *Yale J. Biol. Med.* **13**: 679.
39. FAVOUR, C. B. 1954. Effect of adrenalectomy on the tuberculin reaction in mice. *Proc. Soc. Exptl. Biol. Med.* **85**: 237.
40. BERDET, W. & G. WILDEMANN. 1952. Die Tuberkulinesistenz der Granulocyten als Aktivitätsindex der Tuberkulose. *Beitr. Klin. Tuberk.* **107**: 529.
41. MILLER, J. M. & C. B. FAVOUR. 1951. The lymphocytic origin of a plasma factor responsible for hypersensitivity *in vitro* of tuberculin type. *J. Exptl. Med.* **93**: 1.
42. SANFORD, J. P., C. B. FAVOUR & M. S. TRIBEMAN. 1954. Absence of serum gammaglobulins in an adult. *N. E. J. Med.* **250**: 1027.
43. GOOD, R. A., R. L. VARCO, J. B. ALST & S. J. ZAK. 1957. Transplantation studies in patients with agammaglobulinemia. *Ann. N. Y. Acad. Sci.* **64**(5): 882.
44. PORTER, H. M. 1957. The demonstration of delayed type reactivity in congenital agammaglobulinemia. *Ann. N. Y. Acad. Sci.* **64**(5): 932.
45. WELLS, A. Q. & J. A. H. WYLIE. 1949. A tuberculin neutralizing factor in the serum of patients with sarcoidosis. *Lancet*. : 439.

46. COLE, L. R. & C. B. FAVOUR. 1955. Correlations between plasma protein fractions, antibody titers, and the passive transfer of delayed and immediate cutaneous reactivity to tuberculin, PPD, and tuberculopolysaccharides. *J. Exptl. Med.* **101**: 391.
47. CHASE, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc. Soc. Exptl. Biol. Med.* **59**: 134.
48. SKOG, E. 1955. Experimental studies on hypersensitivity to 2,4 dinitrochlorobenzene and tuberculin in animals. II. Passive transfer of tuberculin sensitivity. *Acta Dermato-Venereol.* **35**: 253.
49. SKOG, E. 1955. Experimental studies on hypersensitivity to 2,4-dinitrochlorobenzene and tuberculin in animals. III. Parabiosis experiments. *Acta Dermato-Venereol.* **35**: 264.
50. MITCHISON, N. A. 1954. Passive transfer of transplantation immunity. *Proc. Roy. Soc.* **142**: 72.
51. LANDSTEINER, K. & M. W. CHASE. 1939. Studies on the sensitization of animals with simple chemical compounds. VI. Experiments on the sensitization of guinea pigs to poison ivy. *J. Exptl. Med.* **69**: 767.
52. HAGERMAN, G. 1954. How is epidermal hypersensitivity transmitted through lymphocytes? *Acta Dermato-Venereol.* **34**: 51.

Discussion of the Paper

CHANDLER A. STETSON (*New York University College of Medicine, New York, N. Y.*): It has been pointed out by several investigators that the concentration of tuberculoprotein required to elicit effects *in vitro* is frequently far higher than that required to produce severe local or systemic effects *in vivo*. Furthermore, many of the early and subsequent tissue-culture observations revealed effects that were demonstrable only after incubation of the tissue in tuberculin-containing media for 2 or 3 days or even longer, as contrasted with the relatively shorter time required for the *in vivo* effects.

Another puzzling feature is that, while the statement is commonly made that tuberculoprotein selectively damages the cells of hypersensitive donors, the experimental observations reported usually show that tuberculoprotein is toxic for normal tissues as well, and that there is only a relatively narrow range over which the tuberculous tissue is damaged and the normal controls not damaged. In fact, it is the exception rather than the rule to find in the literature an experiment in which a given concentration of tuberculoprotein has been found to exert a profound cytotoxic effect on tuberculous tissue and no effect whatever on normal control tissue.

Furthermore, there appears to be a qualitative as well as a quantitative similarity between the effects of tuberculoprotein *in vitro* on normal and tuberculous cells. That is, qualitatively the same changes observed in tuberculous explants exposed to tuberculoprotein can be demonstrated in normal tissues merely by increasing the dose of tuberculoprotein. It is difficult, under our present mode of thinking, to conceive of normal tissues as being only somewhat less hypersensitive to tuberculoprotein than those of a tuberculous donor.

Taken together, these points suggest that perhaps more consideration should be given to the possibility that the *in vitro* effects of tuberculoprotein represent a real set of phenomena that may, however, have no relation to the existence of delayed hypersensitivity or bacterial allergy in the donor. The tuberculous or sensitized host certainly differs from its normal control in many ways other than in the possession of delayed hypersensitivity, and it may not be advisable to attribute real significance to the circumstantial correlation between the allergic state and the *in vitro* phenomena discussed by Favour.

PASSIVE IMMUNITY AGAINST FOUR MOUSE LEUKOSES BY MEANS OF ISOIMMUNE SERA*

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The host response to homografted tissue is complex. Similarities between transplantation immunity and bacterial immunity suggest that some mechanisms involved are common to both, and that the use of classical immunological methods may be of value in analyzing the participants. The relative importance of the individual factors varies with the nature of the graft and the genetic relationship between the donor and the recipient. The most readily available component for study is antibody. Frequently the evidence indicating the importance of a given component may be derived only indirectly. In this way procedures that are known to affect antibody production against bacterial or other antigens will also affect the fate of grafted tissue, either neoplastic or normal.^{1, 2, 3, 4} In some cases, antibody is capable of producing an effect independent of any other mechanism, leaving the host apparently unaffected by its exposure to the foreign tissue. The object of this paper is to summarize the results of recent experiments with passively transferred antibody.

The experiments fall naturally into 4 groups. The first group provided the background for the studies in passive immunity. The second was performed by one of us (Amos) in collaboration with Gorer, using the C57BL leukemia E.L.4. Some of the results were reported by Gorer to the Academy.⁵ The observations were then extended to include 3 additional tumors. The final group of experiments demonstrated the relative effectiveness of some salt-precipitated fractions against 1 of these tumors, the DBA/2 lymphoma (Dalton).

The initial observation was made by Gorer,⁶ who observed some years ago that serum from a hyperimmunized mouse, when mixed with a suspension of tumor cells, would prevent their subsequent growth *in vivo*. Neutralization tests of this type have been repeated with different species and with different tumors.^{7, 8, 9} Tumor neutralization can apparently be performed with relative ease with leukemias and lymphomas, and with considerably more difficulty with some other tissues, notably skin and sarcomata.

The first group of experiments was designed to test the susceptibility of the tumor E.L.4 to immune serum and to determine the fate of the isoantibody when injected into an unimmunized mouse, the antibody level reached, and the rate of its destruction.¹⁰ E.L.4 proved a very suitable tumor for passive immunity experiments in that it produced a very cellular, nonbloody ascites with no clumping of the leukemic cells. It did not kill mice of foreign strains and, following subcutaneous inoculation, it produced easily palpable and measurable nodules that commonly appeared at the 5th day and began to regress on the 9th or 10th. It invariably killed mice of the C57BL strain or of F₁ crosses with C57BL.

* Supported in part by Grant No. DR1R-281 of The Damon Runyon Memorial Fund for Cancer Research, Inc., New York, N. Y.

The antibodies used were prepared in BALB/C, A, or C3H mice by a course of 3 or 4 injections of live cells, the final inoculum being of the order of 50 million cells. The hemagglutinin titers were estimated by the Dextran method,¹¹ and, in most of the experiments, these were over 4000 for A cells.

Neutralization tests, in which it was found that 0.1 ml. of serum would prevent subsequent growth of up to a million cells, were performed.

When antibody was injected into mice, the hemagglutinin level reached in the blood was proportional to the volume injected over the range 0.01 to 0.2 ml. With an injection of 0.1 ml., the dilution appeared to be about 1 in 20, indicating that approximately half the antibody had left the circulation. Daily determinations were made, using different mice on each occasion to avoid loss of antibody through bleeding. The half life was usually of the order of 2 days. A number of the results obtained were shown in FIGURE 1. Simultaneous neutralization experiments confirmed the fact that the cytotoxic activity had also persisted in reasonable amounts for at least 3 days.

The results obtained in this group of experiments were encouraging, and an attempt was made to demonstrate passive immunity.¹²

BALB/C mice that had been inoculated with 0.1 ml. of the antibody BALB/C anti-E.L.4 some hours previously were injected subcutaneously with varying doses of E.L.4. All the experimental animals showed evidence

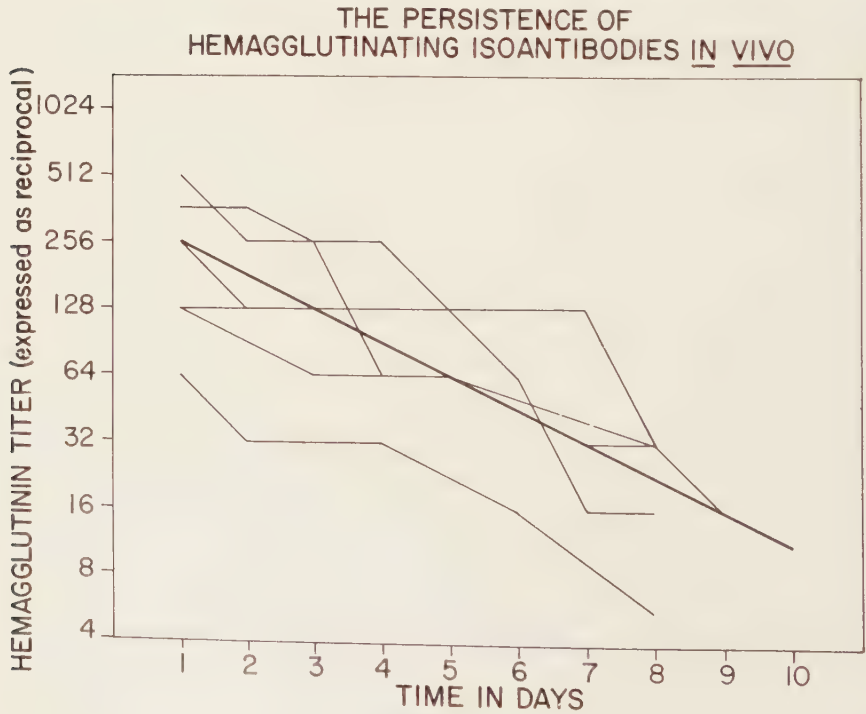


Figure 1

of protection, even when doses of up to 2 million cells were injected, while all the control animals developed nodules.

That this was true passive immunity was shown when the protected animals were given a challenge dose of tumor some weeks later. The previously protected animals now developed tumors, while the actively immunized control animals did not. Furthermore, the hemagglutinin titer in the previously protected group was low, corresponding to that found after primary immunization, whereas in the controls it was high, corresponding to a secondary response.

A number of passive immunity experiments were performed in the A and C3H strains, using fresh, stored, and lyophilized sera, with similar results. The effect was found to last for some time, protection being obtained if the serum was given as long as 7 days before or 2 days after the cell doses. In some experiments the control animals were given up to 0.3 ml. of normal serum (3 times the dose usually injected); in others, animals were treated with antibody prepared in C57BL against an A-strain tumor, and neither of these sera had any inhibitory effect.

Absorption experiments with liver, with fetal tissue, and with E.L.4 cells showed that, while the activity could be lowered by absorption with E.L.4, the other tissues had little effect. This suggested that there might be 2 independent antigens involved: (1) the H-2, evoking antibodies that could be absorbed by the liver; and (2) the X antigen, evoking antibodies that could not be absorbed by the liver.

Passive immunity experiments were therefore carried out in the compatible C57BL mice. Protection could be demonstrated very readily, even though there were no detectable circulating H-2 antibodies. The serum could be injected as long as 3 days before or 1 day after the tumor cells and still give almost full immunity. At greater differences in timing, the effect was less pronounced in these animals than in the resistant mice.

The protection afforded differed from that given to the other strains in that the animals developed progressive tumors when the effects of the serum had worn off. These tumors progressed at the normal rate and eventually killed the mice, so that if the nodules in the treated animals developed 10 days after the controls had become positive, the experimentals would die about 10 days later than the controls.

Further experiments with E.L.4 were designed to test the effects of the 2 types of antibody and to determine if the X antigen was an indication of a previously unsuspected incompatibility between E.L.4 and its C57BL host.

Antibodies prepared against another C57BL tumor or against C57BL liver and spleen would protect in foreign strains, but not in C57BL. Antibodies prepared in BALB/C against an A-strain tumor (sharing the antigen E with C57) would give neutralization against E.L.4; evidently, then, antibodies against the relevant H-2 component could be effective even in the absence of antibodies against the X antigen. A number of mice from the back cross (BALB/C \times C57BL) \times BALB/C were typed for their H-2 antigens and immunized, the animals heterozygous for the antigen H-2B having been previously

TABLE 1
ANTIGENIC STRUCTURE OF H-2 COMBINATIONS USED

Genotype	Strains	H-2 Antigens detected	Probable antigenic constitution	Ascites tumor
H-2 ^a	A/St	ACDEFKH	ACDEFKH	lymphoma No. 2
H-2 ^d	A/Ha BALB/C	CDE ^d FH	aCDE ^d FkH	DBA/2 lymphoma
H-2 ^k	DBA/2			
H-2 ^q	C3H/St	ACEKH	ACdEfKH	6C3HED lymphosarcoma
H-2 ^b	DBA/1	CQEF	aCdEFQh	
	C57BL	D ^b EF	acD ^b EFkh	E.L.4 leukemia
	129 Runner/Jax			

immunized with C57BL blood.¹³ Serum from the mice possessing the H-2B antigen did not, of course, produce antibodies against H-2, and gave very feeble protection even in BALB/C, while the homozygotes lacking the H-2B antigens and producing anti-H-2B gave moderately good protection in both BALB/C and in C57BL. This suggested that there might be a synergistic effect between the production of H-2 antibodies and the production of anti-X antibodies. Attempts to produce a state of active immunity in C57BL mice by tail inoculation and amputation, or by ligation of a subcutaneous tumor, were uniformly negative. Serum from C57BL, or the F₁ with BALB/C, carrying the tumor, showed no protection when used in volumes of up to 0.7 ml., 7 times the serum volume usually employed.

The nature of the X antigen remained unknown. Of the number of possibilities, three seemed to be most likely: (1) that this was an antiviral component; (2) that this was an antigen specific for the tumor E.L.4; or (3) that the antigen was present in all C57BL tissues, but concentrated in the tumor. The last hypothesis appeared the least likely, as antibody against another C57BL tumor had shown no X activity.

It appeared logical to perform similar tests with other tumors in different strains to see if X-type antigens could be detected in other tumors. These experiments made up the third group.

Three ascites lymphomas were available from Hauschka's colony: the C3H lymphosarcoma 6C3HED; the A-strain lymphoma No. 2; and the DBA/2 lymphoma. All were strain-specific and none had ever regressed in the home strain while in Hauschka's colony, although the first 2 had been reported as regressing in some sublines*. Antibodies were prepared in the usual manner, although the final cell doses for immunization were more difficult to obtain, the tumors being less prolific than E.L.4. Experiments were performed, following the same pattern as in the previous group, to attempt to demonstrate passive immunity in both the home strain and in resistant strains. The antigenic pattern of these tumors and the mouse stocks is shown in TABLE 1.

With each of these 3 tumors, passive immunity could be demonstrated readily, although there were differences in detail from the experiments per-

* As of November 1956, 6C3HED and lymphoma No. 2 were found to have regressed occasionally in the home strain after subcutaneous inoculation. Sometimes the C3H mice succumbed to subsequent intraperitoneal challenge of 6C3HED.

formed with E.L.4, and some of the methods previously employed needed modification.

In resistant strains, solid immunity could be obtained against cell doses of up to 2 million cells, and the protected mice appeared to be unaffected by their exposure to the tumor. Antibody titrations carried out following subsequent reinoculation with tumor gave no detectable antibody until about the ninth day, and the titer obtained then was comparable to that obtained with control mice that had not been previously exposed. This was identical with the results obtained with E.L.4, and contrasts with the transfer of adoptive immunity.¹⁴

The home strain could also be protected by antibody given as long as 24 hours before inoculation with tumor cells, but with each of these tumors the immunity was effective against fewer cells and was usually of shorter duration than with E.L.4. This may be due in part to the more prolonged incubation period of the smaller inocula. Protection could consistently be obtained against cell doses in the order of 30,000 to 50,000 cells, and usually lasted for about 3 days; tumors appeared in the control animals on the 6th or 7th day and in the protected group on the 9th or 10th day. The protective period could be significantly prolonged by a second injection of antibody given before tumors appeared in the controls.

A summary of results against these tumors and a comparison with E.L.4 is given in TABLE 2. While the proportion of mice of resistant strains successfully protected agrees closely with the figures for E.L.4, the figures for protection in the home strain are somewhat less consistent. This is because some sera, although of similar hemagglutinin titer, have proved less effective than others. The reason for this is still under investigation, but it appears probable that some sera are relatively unstable when kept at -43°C . and also *in vivo*, where the half life may be only about half that previously found.¹⁰

A number of attempts have been made to induce cross protection with different tumors, including some not previously mentioned. While protection has proved possible with the H-2 antibodies, no cross reactions have yet been demonstrated, either in protection or in absorption experiments, with antibodies against the X-type antigens. These experiments are being continued, as are a series of experiments designed to test the relative effectiveness of a number of the H-2 antigens.

The final group of experiments was performed by the authors, and involved

TABLE 2
PROTECTION AGAINST FOUR TUMORS BY ISOIMMUNE SERUM

Tumor	Antibody produced in	Susceptible mice		Resistant mice	
		Protected	Control	Protected	Control
6C3HED	C57 or 129	1/11	12/12	0/17	16/18
DBA/2 lymphoma	C57 or DBA/1	5/12	12/12	4/21	15/15
A No. 2 lymphoma	C57 or C3H	8/23	23/23	0/27	24/25
E.L.4	A, BALB/C or C3H	3/54	36/37	2/88	41/43

Numerator indicates number of mice developing tumor. Denominator indicates number of mice tested

the fractionation of one antibody—that produced in DBA/1 mice against the DBA/2 lymphoma. This combination was chosen because these mice were derived from a common stock (the most conspicuous difference being at the H-2 locus), and because the DBA/1 mice regularly developed extensive nodules after inoculation with DBA/2 lymphoma cells, thus facilitating measurement.

Precipitation with sodium sulphate was employed. The antibody was collected, a sample taken for titration, and the remainder fractionated as soon as possible after collection. When appreciable delay occurred, the protein fractions appeared to lose much of their activity. The fractions were lyophilized and stored in a refrigerator until required, and then reconstituted. The effective dose appeared to be approximately 1 mg. dry weight of protein fraction per mouse, but the effect was augmented if a second injection of about 0.7 mg. was given 4 to 6 days later.

A series of 6 sets of experiments has been made with fractionated antisera. In preliminary experiments, 4 fractions were tested; these were successively precipitated at 12, 13.5, 18, and 20.3 per cent sodium sulphate. In some experiments, only 2 fractions have been made, precipitated at 13.5 and 18 per cent, as these were found to contain all the hemagglutinating and protective activity. The other fractions were inert by themselves, although no attempts have been made as yet to determine if they exert an effect on the 2 active fractions. The loss of activity during fractionation was high. Over 200 mice were used for antibody production.

The 18 per cent fraction includes the alpha and beta globulins and has little hemagglutinating activity. This fraction will protect in both DBA/2 and in DBA/1, and appears to contain antibodies against the X-type antigens.

The 13.5 per cent fraction has high hemagglutinating activity and will protect in DBA/1 mice. Only feeble protective action can be seen in DBA/2, and this is almost certainly due to contamination with the alpha beta fraction. The 13.5 per cent fraction includes the gamma globulin and is the antibody directed against the H-2 antigens.

The results of 3 of the experiments are shown in TABLE 3. The results of the other 3 experiments were in agreement with these, but the differences were less clearly defined owing to inadequate dosage or loss of activity during preparation.

Two of the findings in the experiments reported above need some amplification: the demonstration of passive immunity against ascites tumors in 4 strains

TABLE 3
PROTECTION AGAINST THE DBA/2 LYMPHOMA BY SODIUM SULPHATE PRECIPITATED
FRACTIONS OF ANTIBODY PRODUCED AGAINST IT IN DBA/1 MICE

Precipitated by Na ₂ SO ₄ (%)	Principal component	Number* developing tumor in	
		DBA/2	DBA/1
0-13.5	Gamma globulin	8/10	0/10
13.5-18.0	Alpha + beta globulin	1/10	1/10
18.0-20.5	Albumin	10/10	9/10

* Numerator indicates number of mice developing tumor. Denominator indicates number of mice tested.

of mice, and the demonstration of different, apparently unrelated, X-type antigens.

The ease with which passive immunity can be provided against the ascites lymphomas strongly suggests that, for the destruction of this type of tumor, antibody may be the most active single component. Gorer has demonstrated that hemagglutinin appeared regularly 4 days after implantation of tumor cells and that this was followed by a fall shortly before regression and a rapid rise after the tumor starts to regress.¹⁵ Although the concentrations detected are small, needing special techniques for their demonstration, it appears probable that the tumor cells are progressively affected by continuous exposure to antibody. In many other cases, the reaction is almost certainly more complex, and it is possible that immune cells can produce a local concentration of sessile antibodies that augment the circulating antibody to bring a critical dose of antibody to the cells.

The almost invariable emergence of a tumor, sometimes after an interval of 2 or more weeks in the protected susceptible animals, and the occasional appearance of a nodule in the protected resistant animals after the tumors have regressed in the controls, suggests that the antibodies may, at the concentrations employed in these experiments, have a cytostatic rather than a cytolytic action. The size of the inoculum required to produce a tumor in a foreign strain further suggests that there are other defense mechanisms that have not yet been studied.

The significance of the X-type antigens is still being examined. Their existence in each of the tumors so far studied and the absence of any evidence of cross immunity suggest that they represent some substance peculiar to the tumor. It appears probable that they have either resulted from a mutation in the tumor or represent antigenic products of a virus being carried in the tumor.

Summary

Antibodies prepared by hyperimmunizing mice of a resistant strain against a leukemia or lymphoma will passively immunize mice of both resistant or susceptible strains.

Two independent antigens appear to be effective in stimulating antibody production.

The antigens of the H-2 system are distributed throughout the animals and the tumor. Antibodies against this system will protect in resistant mice, but not in susceptible mice.

X-type antigens appear to be confined to the tumor. Antibodies against these antigens will protect in any strain. No cross reaction between different X-type antigens has been detected.

The H-2 antibodies are precipitated by 13.5 per cent sodium sulphate, and the X-type antibodies are present in the 18 per cent fraction.

References

1. GOOD, R. A., R. L. VARCO, J. B. AUST & S. J. ZAK. 1957. Transplantation studies in patients with agammaglobulinemia. *Ann. N. Y. Acad. Sci.* **64**(5): 882.

2. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature*. **172**: 603.
3. ODELL, T. T., R. D. OWEN, F. G. TAUSCHE & D. L. LINDSLEY. 1957. The homotransplantation of functional erythropoietic elements in the rat following total-body irradiation. *Ann. N. Y. Acad. Sci.* **64**(5): 811.
4. AMOS, D. B., P. A. GORER, Z. B. MIKULSKA, R. E. BILLINGHAM & E. M. SPARROW. 1954. An antibody response to skin homografts in mice. *Brit. J. Exptl. Pathol.* **25**: 203.
5. GORER, P. A. 1956. The value of ascites tumors in problems of tumor immunity. *Ann. N. Y. Acad. Sci.* **63**(5): 882-891.
6. GORER, P. A. 1942. The role of antibodies in immunity to transplanted leukemia in mice. *J. Pathol. Bacteriol.* **54**: 51.
7. BURMESTER, B. R. 1947. The cytotoxic effect of lymphoid tumor antiserum. *Cancer Research*. **7**: 459.
8. KIDD, J. G. 1946. Suppression of growth of Brown Pearce tumor cells by a specific antibody. *J. Exptl. Med.* **83**: 227.
9. MITCHISON, N. A. 1955. Iso-antibody against a tumor. *Transplantation Bull.* **2**: 93.
10. AMOS, D. B. 1955. The persistence of mouse iso-antibodies *in vivo*. *Brit. J. Cancer* **9**: 216.
11. GORER, P. A. & Z. B. MIKULSKA. 1954. The antibody response to tumor inoculation. Improved methods of detection. *Cancer Research*. **14**: 651.
12. GORER, P. A. & D. B. AMOS. 1956. Passive immunity against C57BL leukemia E.L.4 by means of iso-immune serum. *Cancer Research*. **16**: 338.
13. AMOS, D. B., P. A. GORER & Z. B. MIKULSKA. 1955. The antigenic structure and genetic behavior of a transplanted leukemia. *Brit. J. Cancer*. **9**: 209.
14. MITCHISON, N. A. & O. L. DUBE. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. II. *J. Exptl. Med.* **102**: 179.
15. GORER, P. A. 1956. Personal communication.

THE PROBLEM OF SPECIFIC INHIBITION OF ANTIBODY SYNTHESIS IN ADULT ANIMALS BY IMMUNIZATION OF EMBRYOS*

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This study stems from an attempt to analyze 2 of the basic problems concerning antibody synthesis: (1) that embryos cannot be provoked to form antibody, and (2) that adult animals do not produce antibody against most of their own antigens. Various investigations have shown that the antibody-forming system develops late in the differentiation of the animal.⁷ Only scanty information is available, however, as to how and when, during the period of development, the antibody-forming mechanism acquires the ability to distinguish foreign protein. An interpretation of these phenomena was implied by the results of experiments on acquired tolerance to tissue homotransplantation; it appeared that, during its differentiation, the antibody-forming mechanism passed from a stage in which it was specifically and permanently inhibited by contact with antigen to a stage in which it was specifically induced.⁴

There are 2 ways of analyzing this problem further: (1) one may fractionate the cells used to set up acquired tolerance and attempt to obtain defined components that are responsible for the tolerance state, or (2) one may use foreign antigens and attempt to show that a specific and permanent inhibition of antibody synthesis can be set up during embryonic life. We chose the latter approach because well-defined antigens as well as clear-cut methods for the quantitative analysis of antibody were available to us. Furthermore, a successful demonstration of specific inhibition with defined systems would have greatly simplified the interpretation of acquired tolerance.

When this work was begun, 2 apparently contradictory findings had been published; in both cases, the chicken was used as the test animal.^{8,9} On the one hand, Burnet *et al.*⁸ found no effect, after injection of an antigen during embryonic life, on the ability of the adult to produce antibody to that antigen; on the other hand, Buxton⁹ found such an effect. Since both workers used essentially qualitative methods to determine antibody, the interpretation of any result obtained on the inhibition or lack of inhibition of antibody synthesis is very limited. Furthermore, in Buxton's experiments there was no evidence presented that the inhibition was specific. For these reasons it appeared justifiable to repeat the above experiments by using several antigens, the antibody to which could be measured quantitatively. These included protein (diphtheria toxoid, *Escherichia coli* β -galactosidase, *Escherichia coli* bacteriophage T2), polysaccharide (pneumococcal SSS II), and a hapten diazotized to protein (arsanilic acid-bovine serum albumin). These antigens vary not only in composition but in size, increasing in molecular weight from approximately 10^5 for toxoid and diazoprotein, to 10^6 for the β -galactosidase and SSS II, and to a particle weight of 10^8 for T2 phage. With this range of substances it was

* This work has been supported by grants from the National Science Foundation, Washington, D. C., and the American Cancer Society, New York, N. Y.

hoped that specific inhibition would be revealed. This was not the case, however. Chicken embryos injected with these antigens did not produce any antibody and, when they were challenged 10 to 12 weeks after hatching, their response to the antigen they had received as embryos was equal to that of the controls. This result sets a lower limit to the conditions for revealing a specific inhibition.

MATERIALS AND METHODS

Preparation of Antigens

Diphtheria toxoid. A commercial preparation of toxoid* was found to be 62 per cent precipitable when analyzed with a standard horse antitoxin**, following the procedures described elsewhere.¹⁴ The toxoid (2000 Lf. /ml.) was purified further by precipitating it twice between 35 and 45 per cent of saturation of ammonium sulfate at pH 6.7 and at 4° C. It was then further treated by electrophoresis on starch,²⁰ and a preparation 91 per cent precipitable by antitoxin was isolated.

Atoxic diphtherial proteins (P-proteins). These proteins were prepared by growth of the bacillus on a medium containing a high iron concentration†.

Arsanilic acid-bovine serum albumin (Ars-BSA). Arsanilic acid was diazotized and coupled to crystalline bovine serum albumin‡ at a level of 8 molecules of arsanilic acid per molecule of protein, following the procedure of Pressman *et al.*³⁷ It should be noted that solutions of the diazoprotein are not stable. A gradual breakdown occurs on standing, with release of dialyzable arsanilic acid (or some derivative of it) and loss of color. About 50 per cent of the color is lost at 4° C. in pH 7.0 phosphate buffer after 2 months. For this reason the diazoprotein was prepared fresh every 2 weeks and was dialyzed before use.

Arsanilic acid-chicken egg albumin (Ars-Ea). Prepared exactly as was the Ars-BSA, except that crystalline egg albumin§ was used.

E. coli β -galactosidase. This induced enzyme was purified from a bacterial extract by precipitation of the nucleic acids with streptomycin, followed by alcohol fractionation and, finally, by electrophoresis on a starch column.^{11, 15, 51, 32}

It had a specific activity of 1.1×10^6 units¶ /mg. N. The best preparations which, by physical criteria, are about 80 to 90 per cent pure, have specific activities of 1.6×10^6 units/mg. N. The enzyme used here was, therefore, only 50 to 60 per cent pure.

E. coli bacteriophage T2. A lysate prepared as described elsewhere¹ was treated with deoxyribonuclease and purified by differential centrifugation until the preparation had a constant optical density at 260 m μ of 1.3 per 10¹¹ plaque-forming units. Assuming that Hershey's best preparations²⁷ with a value of 0.9 are pure, the material used here was 80 per cent pure.

* Supplied by the Massachusetts Antitoxin Laboratories, Jamaica Plains, Mass.

** We thank L. Pillemer for generously providing us with standard antitoxin described by Lepow and Pillemer.³⁴

† We are indebted to H. Bowen for this material.

‡ Armour and Company, Chicago, Ill.

§ Pentex Corporation, Chicago, Ill.

¶ One unit equals 1 mM of orthonitrophenol β -D galactoside hydrolyzed per minute at 28° C., pH 7.0 under conditions of saturation by Na⁺ (Armour and Company) and substrate.

Pneumococcus type II D39S. The strain was repeatedly passed through mice until the minimum lethal dose remained constant at 5 to 10 colony formers. The freshly isolated organisms were grown aerobically in neopeptone broth, to which was added 5 per cent horse serum, 0.5 per cent glucose, and 10 $\mu\text{g./ml.}$ crystalline horse-liver catalase. When the pH was 6.3, the exponentially growing culture was cooled to 0° C., and washed 5 times with 0.02 M potassium-phosphate buffer pH 7.2 in 0.85 per cent sodium chloride. No neutralization of the culture was attempted in order to avoid polysaccharide degradation;²⁵ therefore, yields of bacteria were about 20 per cent of those maximally attainable. The washed suspension was heated to 65° C. for 30 minutes, formalin was added to a final concentration of 0.2 per cent, and the preparation was stored at 4° C. for 2 days. It was then washed twice with buffer to remove any unreacted formalin and lysed bacteria. The suspension was sterile and gave a uniform gram-positive stain and an excellent Quellung reaction. Preparations stored at 0° C. retained the gram-positive reaction for about 2 months and were used only during that time.

Pneumococcal polysaccharide SSS II. A purified preparation* of polysaccharide containing 2 per cent nitrogen by weight was treated with the Sevag procedure and precipitated twice with alcohol, as described by Kabat and Mayer.³¹ The nitrogen content was reduced to 0.73 per cent, 3 times the value of 0.26 per cent reported for this polysaccharide.⁶ No further attempts to characterize this nitrogen impurity as protein or nucleic acid were made. Since the method of preparation of the original material supplied by the Lederle Laboratories involved a neutralized culture, there could well have been some altered polysaccharide present. This possibility, however, would not be expected to affect the results significantly, since the polysaccharide was used only in the determination of antibody.

Measurement of Antibody Levels

Quantitative analysis of the precipitin reaction with chicken antisera is complicated by the presence of an α -globulin component that coprecipitates with the antigen-antibody complex and that often accounts for 50 per cent of the nitrogen of the specific precipitate.¹⁶ Although a study²¹ on the increased precipitation of chicken antiserum in high salt concentration (8 to 10 per cent) has appeared, this work was carried out in 0.9 per cent NaCl, for it is not yet clear whether or not the increase is due to nonspecific addition of α -globulin. For this investigation it was too cumbersome to fractionate all antisera, so the following compromises were worked out. In the case of diphtheria antitoxin and of anti- β -galactosidase, the respective biological assays described below were standardized against precipitin reactions carried out with one carefully fractionated γ -globulin prepared from pooled antisera. Antibody to the pneumococcal polysaccharide and Ars-BSA is reported as nitrogen specifically precipitated by the corresponding antigen. Since the contribution of the α -globulin fraction was ignored, the results obtained are consistently about 50 per cent higher than the true values. In the case of bacteriophage

* The SSS II (Lab. 35) was very generously supplied by the Lederle Laboratories, American Cyanamid Company, Pearl River, N. Y.

T2, no attempt to relate the first-order inactivation constant to antibody nitrogen was made.

Diphtheria antitoxin. A stable toxin* preparation was titrated in the skin of a rabbit until it no longer gave a reaction. A concentration of toxin 10 times that giving just a detectable reaction was arbitrarily assigned the value of 1 unit, as was the minimum amount of chicken antitoxin that neutralized it. To standardize the neutralization reaction in terms of antibody nitrogen, the following procedure was carried out: seventeen 2-month-old chickens were injected intraperitoneally with diphtheria toxoid (80 Lf. = 37 μ g. protein N) and β -galactosidase (8×10^5 units = 500 μ g. protein N) suspended in emulsion in oil, but without mycobacteria.¹⁴ Three weeks later they were exsanguinated and their sera were pooled. The γ -globulin fraction was isolated by the technique of Deutsch, a small amount of antibody was removed by absorption with P-proteins, and a quantitative precipitin reaction with highly purified toxin† was carried out.¹⁴ For this antitoxin, 1 neutralizing unit was equivalent to 0.0096 μ g. specifically precipitable nitrogen.

The neutralization reaction was carried out observing all of the precautions described by Jerne.²⁵ In order that the titer of different antitoxins be comparable, their avidities must be comparable. For this reason the standard antitoxic γ -globulin was prepared from animals immunized identically with the experimental animals. A random sampling of the sera of the experimental animals was compared to this antitoxic γ -globulin, and it was shown that the avidities for toxin were roughly the same (see Jerne²⁵ for techniques and interpretation) and, therefore, the assays by neutralization tests can be validly reported in terms of antitoxin nitrogen.

Antiarsanilic acid-bovine serum albumin. Two amounts of antigen, 5.1 and 10.2 μ g. N, were added to 1.0 ml. quantities of antiserum. After 2 hours at 37° C. and 2 days at 0° C. the precipitates were washed with cold saline, and the total precipitated nitrogen was determined by Nesslerization. The supernatant fluids were shown to contain excess antibody. From these 2 values, the total antibody nitrogen per ml. was calculated from the Heidelberger and Kendall equation.²⁶

While all of the animals produce antibody to the Ars-BSA, only a small fraction produced antibody specific for the hapten, arsanilic acid. The determination of antiarsanilic acid was carried out, using Ars-Ea instead of Ars-BSA, as above described. One additional control was included, namely the complete inhibition of the precipitin reaction by 0.01 M arsanilic acid neutralized to pH 6.8.

Anti- β -galactosidase. In this case, as in the diphtheria-toxin system, one has a biological activity that can be followed in the antigen-antibody reaction. Since the activity of this enzyme is not affected by antibody, the antibody cannot be titrated by neutralization. A titration can be set up, however, where increasing amounts of enzyme are added to fixed quantities of antiserum and, when precipitation is complete, the supernatants can be analyzed for the

* We are indebted to H. Boyen for this stable toxin prepared in the Massachusetts Antitoxin Laboratories, Jamaica Plain, Mass.

† We are indebted to I. H. Lepow for this toxin preparation No. E-2-2 which, by our analysis, was 93 per cent specifically precipitable.

presence of excess enzyme. The supernatant in which a trace excess of enzyme is first detected defines the correspondence between enzyme and antibody by determining the number of units of enzyme precipitable by a given quantity of antiserum.^{12, 13}

This correspondence was evaluated quantitatively by standardizing an antiserum. The same fractionated preparation of γ -globulin described above was used. Since the quantitative analysis of the precipitin reaction due to β -galactosidase itself would be in error, depending on the extent of other reactions, a small amount of highly purified β -galactosidase was isolated by repeated separation by electrophoresis in starch and by differential centrifugation. This preparation (1.53×10^6 units/mg. N) showed only 1 antigen-antibody reaction by the Oudin analysis¹⁴ at the levels of antigen used here. It was then found that 10^6 units of enzyme ($0.62 \mu\text{g. N}$) were precipitated by $0.72 \mu\text{g. antibody nitrogen}$. All analyses reported here were carried out by determining the maximum amount of enzyme (in activity units) precipitable by a given volume of antiserum and then multiplying this value by a factor ($0.72 \times 10^{-3} \mu\text{g. antibody N per unit of enzyme activity}$) to convert the value to antibody nitrogen precipitated.

Antibacteriophage T2. The neutralizing antibody can best be titrated by determining the first-order inactivation constant of the reaction. This reaction is described by the relationship:

$$\ln \frac{P_0}{P} = ktC$$

where P_0 is initial phage titer, P is residual active phage after a time, t , with a concentration, C , of antiserum, and k is a constant. The conditions under which this reaction could be used to measure antibody were very difficult to establish for the reasons that (1) normal sera, as well as antisera, could activate the phage to titers from 1.5 to 3 times the apparently stabilized value, and that (2) the inactivation rate was not proportional to serum dilution, except in a very narrow range. The first effect was overcome by heating the phage to 65°C. for 3 minutes, as suggested by Sagik,^{3a} then adding 10 per cent heated (56°C. for 30 minutes) normal serum, incubating at 37°C. for 2 hours and then, overnight, at 4°C. Only then were maximum titers reached. Such maximally activated phage preparations gave first-order kinetics with antisera that were heated to 56°C. for 30 minutes to remove the low-level T2 inactivating power present in normal sera. The inactivation rate was proportional to serum dilution only in the narrow range of 20 to 60 per cent inactivation/minute, below which the rate declined and above which inactivation was too rapid to measure accurately. Therefore all sera were diluted so as to fall in this range. The determination was carried out at 37°C. in tryptone-NaCl broth.¹

Antipneumococcal polysaccharide SSS II. This antibody was determined as in the case of the anti-Ars-BSA, except that the initial reaction was carried out with 8.0 and $16 \mu\text{g.}$ of polysaccharide. The absolute values of antibody nitrogen were subject to the same errors as in the case of the Ars-BSA, namely, the coprecipitation of α -globulin.

Since the polysaccharide might have contained some unrelated antigenic

component, it was, of course, important to show that the antibody produced was directed against the polysaccharide itself. The antisera gave a Quellung reaction with the pneumococci and a low-titer agglutination reaction (1/250 to 1/500 serum dilution). Mouse-protection tests were set up, and it was found that about 0.01 ml. of antisera containing 5 to 10 μ g. N precipitable/ml. would protect mice against 100 MLD of pneumococci (700 colony formers). Although this is low, there is distinct protection, since normal sera or sera from control animals gave the same protection at levels of 0.1 to 0.5 ml., representing a tenfold factor. The addition of large amounts of polysaccharide to antisera greatly reduced the protective titer of the antiserum and, therefore, some anti-polysaccharide antibody was present. Thus, either chicken antibody gives lower protection per μ g. antibody than rabbit antibody, or the specific precipitate contains less antibody than estimated. If the α -globulin contributed 50 per cent of the nitrogen in the specific precipitate, these sera contained 2.5 to 5 μ g. antibody N/ml. which, in terms of rabbit antibody, would have protected to a much larger extent. Again, however, the relative values should be comparable.

Immunization of the Embryos

Embryonated eggs derived from the cross between the New Hampshire and either Cornish or Delaware breeds were used*.

The choice of the conditions for injecting the embryos was limited by their low survival rate. Buxton⁹ set 15 to 17 days as the upper limit for the inhibition effect to be demonstrable, and this was used as a guide in this study. At ages earlier than 11 days, the mortality due to the intravenous injection of very small amounts of antigen was about 70 per cent. The diluent itself was without effect. At 14 days, resistance to all of the antigens was increased tenfold to fifteenfold, and the maximum quantities used here resulted in about 50 per cent mortality in the 4 to 5 days following injection. This set the upper limit to the amount of material that could be given. A further loss of about 15 per cent of the animals occurred at hatching or shortly thereafter, so that the final data were collected on about 35 per cent of those animals originally injected.

All of the injections were intravenous. Whenever there was doubt as to the success of the injection, the egg was discarded.

Antigens were diluted in 0.01 M phosphate buffer in saline (pH 7) and were administered in the following doses:

(1) Diphtheria toxoid—192 Lf.	88 μ g. protein N
(2) Ars-BSA	82 μ g. protein N
(3) β -galactosidase— 8×10^4 units*	50 μ g. protein N
(4) T2— 4×10^{10} particles	2.8 μ g. protein N
(5) Pneumococci type 2	50 μ g. total bacterial N

* With large doses of enzyme, death of the embryo occurs, specifically characterized by complete destruction of the cerebellum. With smaller doses, this effect can be eliminated, but the chicks hatch without any down. The result is a chick that appears bald. The down feathers grow out at 4 to 5 weeks. The cerebellar effect appears to be associated with a heat-stable (100° C. 15 min.) component present in *E. coli* extracts from both induced and noninduced organisms and is present even in the most highly purified preparations of enzyme. Once hatched, the chicks showed no toxic reactions to the enzyme preparations even at doses 50 times that given the 14-day embryo.²⁶

* These eggs were supplied by Woods-Mill Hatcheries, Manchester, Mo.

Since our findings are that the injection of antigen into the embryo did not affect the capacity of the adult to produce antibody, only those data obtained by using maximum doses will be reported.

Immunization of Adult Chickens

Before challenge, the chickens were bled. In no instance was antibody detected as a result of the prior injection given during embryonic life. The challenge immunization was given at from 10 to 12 weeks after hatching, at which time the ability to synthesize antibody appears to be maximal. Preliminary experience with graded doses of antigen injected into untreated animals made possible the choice of a dose that evoked an antibody response 50 per cent of the maximal response. Conditions of submaximal dosage were chosen so as to avoid taxing the antibody-synthesizing system, thereby increasing the chances of detecting a weak inhibitory effect of immunization during embryonic life.

Each of the following antigens was given as an emulsion in oil¹⁴ in 1 intraperitoneal injection: diphtheria toxoid, β -galactosidase, and bacteriophage T2. Four weeks later the animals were exsanguinated. Each injection contained either 80 Lf. (37 μ g. N) diphtheria toxoid, 8×10^5 units (500 μ g. N) β -galactosidase, or 10^{11} particles (7 μ g. protein N) bacteriophage T2.

Ars-BSA was given in 4 intraperitoneal injections on consecutive days, after which the animals were left uninjected for 3 days. Each injection consisted of 1 ml. of a buffered solution of Ars-BSA containing 165 μ g. N/ml. This schedule was continued for 3 weeks. The chickens were bled one week after the final injection.

The schedule for immunization with pneumococci was identical to that for Ars-BSA except that the bacteria were injected intravenously, and the dosage was 2×10^8 colony formers (33 μ g. N) per injection. The reason for using whole pneumococci instead of polysaccharide for the immunization of both the embryos and adults is the report of Schmidt and Wolfe³⁹ that the isolated polysaccharide is nonantigenic for the chicken.

The general design of the experiment was as follows:

Five groups of 14-day-old embryos were each injected with a given antigen. Ten to 12 weeks after hatching, each group was challenged with 2 antigens, the same one administered during embryonic life and one other. Although uninjected as well as injected controls were included in the original experiment, the nature of the results makes it unnecessary to include these in the data, for each group turned out to be the best control for the other. It may be added that, in the uninjected controls, no effect on the antibody response to any one antigen was observed when the 2 antigens were injected simultaneously.

The experimental plan is summarized in TABLE 1.

RESULTS

Data comparing experimental with control animals are summarized in TABLE 2. Each animal was injected during embryonic life with 1 antigen and, as an adult, was challenged with 2 antigens—the same one that was given the em-

TABLE 1

Group	Antigen given to embryo	Heterologous antigen administered together with a homologous one to adult chickens	Antibody data found in TABLES
I	Diphtheria toxoid	T2	3
II	Ars-BSA	T2	5
III	β -galactosidase	One half of group received pneumococci; other half received Ars-BSA	6
IV	T2	Diphtheria toxoid	4
V	Pneumococci	One half received β -galactosidase; other half received Ars-BSA	7

TABLE 2

THE SUMMARY OF DATA ON ANTIBODY RESPONSES TO HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

	Experimental animals	Control animals
I. Diphtheria toxoid Antitoxin titer, μ g. N/ml. Number of animals	8.2 ± 4.9 (13)	8.9 ± 6.5 (27)
II. Arsanilic acid BSA Anti-Ars-BSA, μ g. N/ml. Anti-Ars-Ea, μ g. N/ml. Number of animals	44 ± 25 3.3 ± 2.0 (15)	35 ± 22 2.5 ± 1.6 (13)
III. β -galactosidase (Gz) Anti-Gz, μ g. N/ml. Number of animals	31 ± 19 (18)	38 ± 21 (11)
IV. Bacteriophage T2 Anti-T2, min. ⁻¹ ml. ⁻¹ Number of animals	14 ± 11 (27)	18 ± 14 (28)
V. Pneumococcal Polysaccharide Anti-SSS II, μ g. N/ml.* Number of animals	11 ± 10 (15)	8.9 ± 4.0 (10)

The summary of results was calculated from the detailed data on each animal given in TABLES 3 to 7. See text for description of table.

* The absolute values are in error due to the contribution of α -globulin to the specific precipitate.

bryo and another. When considering the antibody response to an antigen given during embryonic life, the animal is referred to as an experimental animal. When considering the antibody response to the second antigen *not* given to the embryo, the same animal is referred to as a control animal. For example, group I experimental animals received diphtheria toxoid as embryos and were challenged at 10 weeks with toxoid. The individual antibody levels are given in column 1, TABLE 3. The control animals for group I are found in group IV, which received T2 as embryos and toxoid as adults. These data appear in column 2, TABLE 4. The average titer and mean deviation for the antitoxin levels in experimental group I is $8.2 \pm 4.9 \mu$ g. N. ml.; for the control group I (or group IV) this value is $8.9 \pm 4.9 \mu$ g. N. ml. Similarly, each group is a control for the other, and the data for TABLE 2 were calculated by averaging the individual serum titers for the homologous and heterologous antibodies and comparing them as experimental and control series.

Under the present experimental conditions, no specific inhibitory effect of

TABLE 3

ANTIBODY LEVELS IN CHICKENS INJECTED AS EMBRYOS WITH TOXOID, AND CHALLENGED AS ADULTS WITH TOXOID AND T2

Serum no.	Antitoxin	Anti-T2
	$\mu\text{g. N}$	$\text{K min.}^{-1} \text{ ml.}^{-1}$
54	5.9	13
56	8.7	51
61	0.43	0.87
62	3.8	12
69	11	14
71	14	19
184	21	100
186	0.13	3.3
187	4.2	6.1
189	3.1	1.3
190	16	8.5
191	9.8	7.3
192	7.6	6.5

TABLE 4

ANTIBODY LEVELS IN CHICKENS INJECTED AS EMBRYOS WITH T2 AND CHALLENGED AS ADULTS WITH T2 AND TOXOID

Serum No.	Anti-T2	Antitoxin
	$\text{K min.}^{-1} \text{ ml.}^{-1}$	$\mu\text{g. N/ml.}$
122	14	3.5
124	45	8.2
125	1.7	15
126	11	20
128	8.7	1.5
129	26	10
130	3.7	9.3
131	13	4.3
132	90	2.3
133	3.6	19
134	3.9	15
135	16	18
136	18	7.4
137	1.5	0.32
138	0.50	11
139	11	0.81
140	17	37
141	19	5.7
143	7.1	13
177	0.31	0.13
182	31	0.87
317	9.1	6.1
318	1.1	2.2
321	3.7	2.7
322	1.4	13
323	3.6	12
411	6.9	1.8

TABLE 5

ANTIBODY LEVELS IN CHICKENS INJECTED AS EMBRYOS WITH ARS-BSA AND CHALLENGED AS ADULTS WITH ARS-BSA AND T2

Serum No.	Anti-Ars-BSA	Anti-Ars-Ea	Anti-T2
	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$	$\text{K min.}^{-1} \text{ ml.}^{-1}$
194	53	Trace	63
195	11	"	10
196	27	"	15
197	33	5.3	5.3
198	100	10	0.80
199	16	Trace	6.7
200	63	"	18
202	70	8.3	31
214	56	2.2	26
215	38	4.7	21
218	19	Trace	8.3
221	16	"	14
223	7.5	"	20
224	67	6.3	3.3
225	82	13	7.6

an antigen given during embryonic life on the subsequent antibody response of the adult could be demonstrated (TABLE 2). The inability to establish a specific inhibition of antibody synthesis may be due to one or both of two factors: (1) the experimental conditions might not be correct, or (2) there might be no stage in the differentiation of the antibody-forming system susceptible to specific inhibition. The distinction between these two possibilities depends in part on the degree to which our results are extended. At present there are 3 independent investigations of this phenomenon in chickens: that of Burnet *et al.*,⁸ that of Buxton,⁹ and our own. Burnet *et al.*⁸ could not demonstrate a specific inhibitory effect in chickens by inoculation *in ovo* with *E. coli* bacteriophage C₁₆ (closely related to T2), human red blood cells, or an active influenza type A virus. These workers injected: (1) 10^{10} particles (0.7 $\mu\text{g.}$ protein N) C₁₆ into the yolk sac of 6-day-old embryos; (2) 2.5×10^8 human red blood cells (120 $\mu\text{g.}$ protein N) intravenously into 11-day-old embryos; (3) 2.5×10^9 human red blood cells (1200 $\mu\text{g.}$ protein N) in the yolk sac of 6-day-old embryos; and finally (4) active influenza virus type A into the allantoic membrane, where extensive but abortive multiplication took place so that the embryos survived and hatched. The amounts of material injected are comparable to those used by us, although the routes are different. The case of human red blood cells is particularly interesting, since normal adult fowl serum contains an antibodylike factor that agglutinates human red blood cells. This factor is absent from the serum during the first 5 weeks after hatching. The production of this normally occurring antibodylike factor was not inhibited by the injection of human cells *in ovo*. Buxton,⁹ using *Salmonella pullorum* as an antigen in embryos, at levels of 0.05 $\mu\text{g.}$ bacterial nitrogen, carried out similar experiments that appeared to demonstrate a specific inhibitory effect. Buxton pointed out that the difference between his and Burnet's results was probably due to the route of injection, since Burnet *et al.*, except in one experiment, used the allantoic or yolk-sac route that he found not to be as successful as the intravenous

TABLE 6

ANTIBODY LEVELS IN A GROUP OF CHICKENS INJECTED AS EMBRYOS WITH β -GALACTOSIDASE (Gz), ONE HALF OF WHICH WAS CHALLENGED WITH β -GALACTOSIDASE AND PNEUMOCOCCI TYPE 2 AND THE OTHER HALF WITH β -GALACTOSIDASE AND ARS-BSA

Serum No.	Anti-Gz	Anti-SSS II	
	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$	
114	57	6.7	
151	71	15	
157	11	8.3	
161	14	Trace	
162	22	6.9	
166	6.8	11	
171	53	9.5	
175	17	23	
325	31	5.4	
326	36	7.5	

	Anti-Gz	Anti-Ars-BSA	Anti-Ars-Ea
	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$
112	44	32	Trace
113	17	26	"
115	8.8	5.8	5.3
153	15	16	8.1
154	91	7.4	Trace
155	15	41	3.5
159	24	19	Trace
162	18	81	"

TABLE 7

ANTIBODY LEVELS IN A GROUP OF CHICKENS INJECTED AS EMBRYOS WITH PNEUMOCOCCI TYPE 2, ONE HALF OF WHICH WAS CHALLENGED WITH PNEUMOCOCCI AND β -GALACTOSIDASE (Gz) AND THE OTHER HALF WITH PNEUMOCOCCI AND ARS-BSA

Serum No.	Anti-SSS II	Anti-Gz	
	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$	
78	14	59	
79	2.3	77	
82	5.8	21	
84	11	25	
85	13	27	
86	7.6	<0.5	
89	7.9	37	
90	4.3	16	
92	Trace	87	
94	"	48	
97	18	23	

	Anti-SSS II	Anti-Ars-BSA	Anti-Ars-Ea
	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$	$\mu\text{g. N/ml.}$
225	24	92	11
226	<0.5	54	5.5
229	12	61	Trace
107	17	22	"
108	32	3.4	"

route. This argument, however, may not be sufficient, since the influenza virus, which underwent multiplication with resultant cell lysis, would be expected to have been carried everywhere in the egg. Unfortunately, Burnet *et al.*⁸ measured antibody by methods that would have distinguished only very large differences and, with a system as complex as this, the adult animals would not be expected to give all-or-none responses.

Buxton tried to refine the antibody determination, and used a qualitative agglutinin test. The antiserum was diluted until an arbitrary end point of agglutination of bacteria was reached. In comparing 2 sera, it was assumed that the end point reached was a linear function of the amount of antibody protein present. For example, if one antiserum agglutinated to a 1/5000 dilution and another to 1/100 dilution, the presumption was that the first serum had 50 times more antibody than the second. It should be pointed out, however, that this relationship has been demonstrated only for encapsulated pneumococci⁹ in which the only reactive antigen is the polysaccharide. For *Salmonella*, not only is there no evidence of proportionality between antibody concentration and the dilution of serum that agglutinates the bacteria at any given end point but, due to the antigenic heterogeneity of the surface of this bacterium and due to the presence of nonagglutinating antibodies of widely different avidities (assumed to be directed against the same surface component), it would be surprising to find such a proportionality. The use, then, of dilution titers makes it difficult to evaluate the significance of Buxton's data.

At present, then, we can say only that in chickens no specific-inhibitory effect has been unequivocally demonstrated. That this failure might be due to the lack of a more prolonged contact with the antigen during embryonic life seems dubious from some recent work of Green and Lorincz.²⁰ These workers injected chick embryos with mouse ascites cells that proliferate and extensively invade all tissues. The ascites-cell invasion comes to a halt near hatching, and the chick emerges almost free of these cells. At about 2 months, the experimental chicks were found to produce as much antibody against these cells as the noninjected control animals. This observation raises the question of acquired tolerance that is analyzed in the discussion.

DISCUSSION

The general problems of the interrelationships between acquired tolerance,³ delayed hypersensitivity,⁴ immune paralysis,¹⁹ and immunologic unresponsiveness^{10, 17, 23} have been discussed in detail by the above-cited authors. There is accordingly no need to enlarge on this aspect here.

It is clear that one cannot put too much weight on our negative results by themselves. Only in the light of the totality of the evidence can we see how meaningful is this failure to demonstrate a specific inhibition of antibody synthesis. Therefore I should like to discuss these results simultaneously at two levels: (1) interpretation at the technical level of the apparent contradictions between these and other findings; and (2) evaluation of what is known about the phenomenon of acquired tolerance and specific inhibition.

In order to distinguish the different types of experiments, the term *acquired*

tolerance will be used to describe those experiments in which an embryo or newborn chick is given a cell suspension and challenged as an adult with a tissue homograft. The term *specific inhibition* will be applied to those experiments in which an embryo is injected with a defined antigen, and the response of the adult to a challenge by the given antigen is quantitatively estimated in terms of a defined antigen-antibody reaction.

We might begin by pointing out that successful tissue homografts have been carried out on adult chickens injected during embryonic life.⁴ Thus one can set up a state of acquired tolerance in this species. The failure to demonstrate a specific inhibition of antibody synthesis in adults by injection of defined antigens into the embryo indicates that there may be a need to analyze again the hypotheses concerning acquired tolerance and specific inhibition.

The supposition, reasonable as it is, that the rejection of a homograft involves an antigen-antibody reaction, is based on indirect and limited evidence. Unfortunately, this introduction of uncertainty is not too helpful to investigations in the field, for the moment we reject the hypothesis that antibodies are involved we are faced with providing another hypothesis to account for both the specificity and induction properties of the system. In order to do this, we find ourselves postulating the existence of an induced specific combining substance so like antibody as to be identical with it. Therefore, for the sake of this analysis only, we shall retain the essential assumption that antibody is involved in the phenomenon of acquired tolerance, and we can turn to the question of the mechanism of the phenomenon.

A state of acquired tolerance or of specific inhibition is said to exist whenever a homograft "takes" or whenever no antibody is detectable in the serum after the adult is challenged. This state could obtain either (1) because the antibody-forming mechanism is specifically inhibited, or (2) because the antibody, once formed is specifically neutralized and prevented from appearing in the circulation. This distinction is essential to our understanding of these phenomena and leads to an analysis of the other cases of specific inhibition at present available.

In contradistinction to our negative results with chickens, several groups of workers (Hanan and Oyama,²⁵ Dixon and Maurer,¹⁷ and Cinader and Dubert¹⁰) have shown clearly that injection of newborn rabbits with a defined protein antigen results in a lasting and specific inability of the adults to produce antibody detectable in the serum. Immediately one might ask whether or not we are merely dealing with species difference as regards the ability of a soluble antigen to set up a state of specific inhibition. It would be unsatisfactory to stop at this conclusion, especially since the state of acquired tolerance has been established in both chickens and rabbits, and the state of specific inhibition has been established in rabbits. Implicit in the admission of the species difference as an argument for the failure of our experiments is the assumption that acquired tolerance and specific inhibition involve fundamentally different mechanisms, but we started by rejecting this notion. One wonders, therefore, if any other factors equally important can be found; two suggest themselves:

(1) *The antigen.* From the general observation that acquired tolerance is

most successfully established between individuals of the same species,⁴ it is suggested elsewhere in these pages that the major distinction between the experiments with rabbits and those with chickens lies in the antigens. Specific inhibition was successfully established with rabbits injected with bovine and human serum albumins while, in our case, diazobovine serum albumin and diphtheria toxoid failed to establish a state of specific inhibition in chickens. The argument is that bovine and human serum albumins are more closely related to rabbit protein than are diazobovine serum and diphtheria toxoid to chicken protein. This reasoning by analogy from acquired tolerance to specific inhibition, however, may be misleading since the rejection or acceptance of a tissue graft involves many more than one antigen. As one attempts to establish a tolerant state between increasingly distant species, not only do homologous antigens become more distinct, but many new antigens come into play, since antibody to any of them is capable of provoking a graft rejection. Therefore, it is just as probable that the larger number of completely unrelated antigens, rather than the greater difference between homologous antigens, is responsible for the increased difficulty encountered in setting up a tolerant state between more distantly related species. From the moment that one can no longer measure a cross reaction⁴¹ it is operationally meaningless to say that human serum albumin is more closely related to rabbit serum albumin than is diphtheria toxoid. At present, then, there is no evidence that enables us to evaluate the importance of the source of antigen in either acquired tolerance or specific inhibition.

(2) *The dosage and schedule of injection.* This appears to be the major difference between the work with the two species. In studies on rabbits, relatively large doses of antigen (20 to 1000 mg.) were given by repeated injection, whereas only one injection of antigen (0.1 to 0.5 mg.) was given the chick embryo. In the case of the rabbits, 20 mg. may not represent the lower limit, but good data are not yet available. In the case of the chickens, in order to account for the negative findings, one might say that the amount of antigen used was insufficient to establish a specific inhibition. In the latter case, moreover, there is relative insensitivity of the mechanism of specific inhibition as compared to that of specific induction of antibody synthesis for, on a weight basis, it takes 100 times more antigen to inhibit the newborn rabbit than to induce the adult to form antibody. What could this relative insensitivity mean?

At the beginning of the discussion, the distinction was made between (1) the specific inhibition of antibody synthesis, and (2) the specific blocking of the appearance of antibody in the circulation. Under the first hypothesis, the antigen must intervene directly in the machinery of antibody formation. Whether the system is inhibitable or inducible depends upon the state of its differentiation, since embryonic systems are inhibited and mature ones are induced. According to this hypothesis, then, one might expect, from the nature of the systems involved, this contrast between the insensitivity of the animal to inhibition and its sensitivity to induction since, in the case of inhibition, all of the antibody-forming cells must be reached whereas, in the case of induction, only a fraction need be reached. Under the second hypothesis, the difference

between the embryonic and adult response, inhibition versus induction, lies not in the state of the antibody-forming mechanism but in some other embryonic factor that enables the antigen to get into tissues and remain there, protected against destruction but capable of removing antibody and being regenerated. This might explain the need for large amounts of antigen to establish inhibition, compared to the quantities needed for induction.

This argument becomes even stronger when we consider that (1) in experiments with acquired tolerance, living cells that could survive in the adult were given to the embryo, and (2) in specific inhibition experiments, the antigens were injected either absorbed on alum or in emulsion in oil, techniques that might favor the resistance of the antigen to elimination.

The second hypothesis is not considered probable by most workers in the field. The general tendency is to separate, as basically distinct phenomena, the acquired tolerance and immunological paralysis found when pneumococcal polysaccharides are injected into adult mice. However, the phenomena of immunological paralysis observed by Felton,¹⁹ of acquired tolerance investigated by Medawar and others;⁴ and of specific inhibition revealed by Hanan and Oyama,²³ Dixon and Maurer,¹⁷ and Cinander and Dubert¹⁰ are all compatible with the theory that the continued presence of antigen in tissues is responsible for the "tolerant" or "inhibited" state. One must assume that, in the newborn, protein antigens in large doses can set up an inhibition analogous to that which polysaccharide can effect in adults, the difference being due to the kind of antigen, that is, to the known persistence in adult tissues of polysaccharide compared to proteins. The tendency is to admit that the Felton phenomenon involves the constant neutralization of antibody, whereas acquired tolerance (or specific inhibition) involves the direct inhibition of antibody synthesis. There is no formal proof, however, that immune paralysis does not involve inhibition of synthesis, or that acquired tolerance does not involve neutralization of antibody. Therefore, both phenomena could well be manifestations of the same basic mechanism. The only experimental evidence to the contrary is the demonstration in immune-paralyzed mice of the presence of polysaccharide⁴⁰ capable of combining with passively given antibody, and the inability to show the same relationship with protein antigens in rabbits that were specifically inhibited at birth.¹⁸ These experiments,¹⁸ however, do not disprove that protein antigens exert an inhibitory effect by their continued presence. The difference might be of a quantitative rather than of a qualitative nature. This quantitative difference has already been observed for polysaccharides versus proteins as inducers of antibody formation.

The work of Green and Lorincz²² supports this second hypothesis in the case of acquired tolerance, for they showed that early massive and prolonged exposure of chicken embryos to the antigens of growing tumor cells is not in itself a sufficient condition for the production of a state tolerant to the tumor antigens. These workers feel that their study supports the idea that "the tolerant condition produced by homologous cells depends on their permanent survival in the host" where, during the period of maturation, such cells liberate antigens.

The analysis of the available evidence on acquired tolerance or specific in-

hibition therefore leads us to conclude that neither of the two basic assumptions, neutralization or inhibition, has been eliminated. We are at once led to the rather remarkable symmetry between the problem of inhibition and the problem of induction of antibody synthesis. The two major hypotheses concerning antibody synthesis differ in one aspect. Theory 1 (the template theory) of Breinl and Haurowitz,⁵ Mudd,³¹ Alexander,² and Pauling³⁶ places the antigen directly in the antibody-forming mechanism as a template, so that the origin of the specificity of the antibody is directly due to the antigen*. Theory 2 (the modified enzyme theory) of Burnet and Fenner⁷ places the antigen indirectly in the antibody-forming mechanism by interposing a specifically induced "enzymatic" system that makes the antibody. The first theory implies that the continued presence of antigen is necessary to antibody formation while, in the latter theory, the synthesis of antibody should continue in the absence of antigen. As yet, no definitive experiment has been reported.

The specific inhibition of antibody synthesis by embryonic contact with antigen is a symmetrical analogue of specific induction, but it is not a necessary consequence of either theory. To account for a specific inhibition, one can make an additional hypothesis, as did Burnet, as to the existence of a specific self-marker system formed by contact with antigen during embryonic or early life, and responsible for the recognition of antigens present during embryonic or early life. Such an assumption can be made under either theory. Thus reasoning by analogy with the theories on specific induction, under one theory the "self-marker system" becomes a manifestation of the continued presence of antigen in tissues while, under the other, the "self-marker system" becomes an altered antibody-forming mechanism excluded from making certain configurations of antibody, the alteration occurring most readily by contact with antigen during embryonic life. As with the induction of antibody synthesis, the inhibition of antibody synthesis poses exactly the same problem as to the role of the antigen. Just as the prolonged production of antibodies to pneumococcal polysaccharide has been used as an argument that the continued presence of antigen is essential to the continued production of antibody,⁴² so the Felton phenomenon can be used to argue for a similar role of antigen in the case of specific inhibition or acquired tolerance.

To render this analogy between induction and inhibition operationally useful, we must make a qualification concerning the role of the antigen. In inhibition phenomena the antigen might be considered either an extracellular regenerated filter that prevents antibody from reaching the circulation or an intracellular inhibitor. Of course, in the case of induction, by definition, the antigen is virtually acting intracellularly. By placing the inhibiting antigen in the cell, the distinction between the two hypotheses of inhibition becomes less clear, but we have not yet reached the point at which precise hypotheses to analyze this latter problem become necessary.

* The very interesting natural-selection theory²⁹ of Jerne, for the purposes of this discussion only, can be placed under theory 2, since he assumes that the continued presence of antigen is not necessary to the continued synthesis of antibody.

SUMMARY

The intravenous injection of 14-day-old chicken embryos with a single dose of the following antigens: diphtheria toxoid, *E. coli* β -galactosidase, *E. coli* bacteriophage T2, pneumococci type II, and arsanic acid-beef serum albumin failed to inhibit the ability of the 10- to 12-week-old adult chickens to respond and to produce antibody against these antigens. The meaning of these findings in the light of what is known about acquired tolerance and about specific inhibition in other species has been discussed.

References

- ADAMS, M., Ed. 1950. Methods of study of bacterial viruses. Methods in Medical Research. **2**: 1. Year Book Publishers. Chicago, Ill.
- ALEXANDER, J. 1931. Intracellular aspects of life and disease. *Protoplasma*. **14**: 296.
- BARRET, C. J., JR. & J. A. TRIPP. 1942. A method for adjusting the antigen-antibody ratio prior to the determination of pneumococci antibody nitrogen. *J. Immunol.* **43**: 311.
- BILLINGHAM, R. F., L. BRENT & P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature*. **172**: 1.
- BREINL, F. & F. HAUROWITZ. 1930. Chemical investigation of the precipitate from hemoglobin and antihemoglobin serum and remarks about the nature of antibodies. *Hoppe-Seyler's Z. physiol. Chem.* **192**: 45.
- BROWN, R. 1939. Chemical and immunological studies of the pneumococcus. V. The soluble specific substances of types 1-32. *J. Immunol.* **37**: 445.
- BURNET, F. M. & F. FENNER. 1949. The production of antibodies. 2nd ed. Macmillan. Melbourne, Australia.
- BURNET, F. M., J. D. STONE & M. EDNEY. 1950. The failure of antibody production in the chick embryo. *Australian J. Exptl. Biol. Med. Sci.* **28**: 291.
- BUXTON, A. 1954. Antibody production in avian embryos and young chicks. *J. Gen. Microbiol.* **10**: 398.
- CINADER, B. & J. M. DUBERT. 1955. Acquired immune tolerance to human albumin and the response to subsequent injections of diazo human albumin. *Brit. J. Exptl. Pathol.* **36**: 515.
- COHN, M. & J. MONOD. 1951. Purification et propriétés de la β -galactosidase (lactose) d'*Escherichia coli*. *Biochim. et Biophys. Acta*. **7**: 153.
- COHN, M. & A. M. TORRIANI. 1951. Étude immunochimique de la biosynthèse adaptative d'un enzyme: la β galactosidase lactose, d'*Escherichia coli*. *Compt. rend.* **232**: 115.
- COHN, M. & A. M. TORRIANI. 1952. Immunochemical studies with β -galactosidase and structurally related proteins of *Escherichia coli*. *J. Immunol.* **69**: 471.
- COHN, M., Ed. 1952. Immunochemical methods for determining the homogeneity of proteins and polysaccharides. Methods in Medical Research. **5**: 268. Year Book Publishers. Chicago, Ill.
- COHN, M. Unpublished results.
- DEUTSCH, H. F., J. C. NICHOL & M. COHN. 1949. Biophysical studies of blood plasma proteins. XI. Immunological and electrophoretic studies of immune chicken serum. *J. Immunol.* **63**: 195.
- DIXON, F. J. & P. H. MAURER. 1955. Immunologic unresponsiveness induced by protein antigens. *J. Exptl. Med.* **101**: 245.
- DIXON, FRANK J., P. H. MAURER & W. O. WEIGLE. 1955. Immunologic activity of pneumococcal polysaccharide fixed in the tissues of the mouse. *J. Immunol.* **74**: 188.
- FELTON, L. D. 1949. The significance of antigen in animal tissues. *J. Immunol.* **61**: 107.
- FLÖDIN, P. & J. PORATH. 1954. Zone electrophoresis in starch columns. *Biochim. et Biophys. Acta*. **13**: 175.
- GOODMAN, M. & H. R. WOLFE. 1952. Precipitin production in chickens. VIII. A comparison of the effect of salt concentration on precipitate formation of pheasant, owl and chicken antisera. *J. Immunol.* **69**: 423.
- GREEN, J. & A. L. LORINCZ. 1956. Growth of mouse tumor in the chick embryo and

- retention of the capacity of the chick to form antibody to the tumor cells in later life. Personal communication.
23. HANAN, R. & J. OYAMA. 1954. Inhibition of antibody formation in mature rabbits by contact with the antigen at an early age. *J. Immunol.* **73**: 49.
 24. HAUROWITZ, F. 1952. The mechanism of the immunological response. *Biol. Rev. Cambridge Phil. Soc.* **27**: 247.
 25. HEIDELBERGER, J., E. A. KABAT & D. A. SHRIVASTAVA. 1937. Cross reactions of types III and VIII pneumococci in horse and rabbit antisera. *J. Exptl. Med.* **65**: 487.
 26. HEIDELBERGER, M. & F. E. KENDALL. 1935. The precipitin reaction between Type III pneumococcus polysaccharide and its homologous antibody. III. A quantitative study and a theory of the reaction mechanism. *J. Exptl. Med.* **61**: 563.
 27. HERSHEY, A. D. 1955. An upper limit to the protein content of the germinal substance of Bacteriophage T2. *Virology*. **1**: 108.
 28. JERNE, N. K. 1951. A study of avidity. *Acta Pathol. Microbiol. Scand. Suppl.* LXXXVII.
 29. JERNE, N. K. 1955. The natural-selection theory of antibody formation. *Proc. Natl. Acad. Sci. U. S.* **41**: 849.
 30. KABAT, E. A. & M. MAYER. 1948. *Experimental Immunochemistry*. Charles C. Thomas.
 31. KUBY, S. A. & H. A. LARDY. 1953. Purification and kinetics of β D-galactosidase from *Escherichia coli*, strain K12. *J. Am. Chem. Soc.* **75**: 890.
 32. LEDERBERG, J. 1950. The β -D galactosidase of *Escherichia coli*, strain K12. *J. Bacteriol.* **60**: 381.
 33. LEPOW, I. H. & L. PILLEMER. 1952. Studies on the purification of diphtherial toxin. *J. Immunol.* **69**: 1.
 34. MUDD, S. 1932. A hypothetical mechanism of antibody production. *J. Immunol.* **23**: 423.
 35. NEUMAN, L. Unpublished results.
 36. PAULING, L. 1950. Theory of the structure and process of formation of antibodies. *J. Am. Chem. Soc.* **62**: 2643.
 37. PRESSMAN, D., M. SIEGEL & L. A. R. HALL. 1954. The closeness of fit of antibenzoate antibodies about haptenes and the orientation of haptenes in combination. *J. Am. Chem. Soc.* **76**: 6336.
 38. SAGIK, B. P. 1954. A specific reversible inhibition of bacteriophage T2. *J. Bacteriol.* **68**: 430.
 39. SCHMIDT, M. H. & H. R. WOLFE. 1953. Precipitin production in chickens. IX. A quantitative study of the antibody response to nine different purified substances. *J. Immunol.* **71**: 214.
 40. STARK, O. K. 1955. Studies on pneumococcal polysaccharide. II. Mechanism involved in production of "immunological paralysis" by type I pneumococcal polysaccharide. *J. Immunol.* **74**: 130.
 41. MAURER, P. H. 1952. The cross reactions between albumins of different species and globulin of different species. *J. Immunol.* **72**: 119.

PRODUCTION OF DELAYED SENSITIVITY TO PROTEIN WITHOUT DETECTABLE CIRCULATING ANTIBODY*

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Bacterial infections, particularly such chronic infections as tuberculosis, generally induce a state of hypersensitivity in the host, whether man or animal. Intradermal injection of specific bacterial proteins into sensitized animals results in the delayed appearance of inflammatory lesions that closely resemble and, indeed, may often be indistinguishable (both grossly and microscopically) from lesions of the Arthus type.¹ Nevertheless, the 2 reactions may be differentiated from one another by the fact that only the Arthus type can be passively transferred to nonsensitive animals with serum from sensitive donors, whereas transfer of "tuberculin"-type hypersensitivity to normal animals can be effected only by injection of large numbers of leukocytes from the sensitive donors.²

Delayed hypersensitivity may be induced in laboratory animals following injection of soluble antigens incorporated in oil emulsions containing killed *Mycobacteria*³⁻⁴ or certain fractions of tubercle bacillus wax.⁵ While animals sensitized in this way show delayed skin reactions following intradermal challenge with specific antigen, the interpretation of these reactions is usually obscured by the presence of precipitating circulating antibody in high titer, a condition known to favor development of Arthus-type reactions. When sufficient numbers of washed leukocytes from animals sensitized in this way are injected into normal animals, however, the recipients may show delayed skin reactions without demonstrable circulating antibody.⁶

Induction of hypersensitivity in guinea pigs by infection with diphtheria bacilli. The diphtheria toxin-antitoxin system is particularly well-suited for experimental studies on hypersensitivity of the delayed type because both toxin and toxoid are well-characterized highly antigenic proteins and because both toxin and antitoxin can be detected and determined quantitatively in minute amounts. Moreover, it has been observed that guinea pigs injected intradermally with living toxigenic diphtheria bacilli, followed a few hours later by a protecting intraperitoneal dose of horse antitoxin (according to the usual "virulence" test), develop hypersensitivity to diphtherial proteins. Small amounts of purified diphtheria toxoid (3 μ g. or less), injected intradermally into guinea pigs 2 to 3 weeks after the infecting dose, elicit marked inflammatory reactions of the delayed tuberculin type. That sensitivity is directed specifically against the toxoid component itself is shown by the fact that markedly reduced reactions are produced by injection of supernatants from which all of the toxoid has been removed by specific precipitation with excess human antitoxin, but which still contain traces of other diphtherial proteins. Despite

* This study was aided by a grant from the National Institutes of Health, United States Public Health Service, Bethesda, Md.

† Aided by a fellowship from the Dazian Foundation for Medical Research, New York, N. Y.

‡ Affiliated also with the National Microbiological Institute, National Institutes of Health, United States Public Health Service, Bethesda, Md.; and the Rocky Mountain Laboratory, Hamilton, Mont.

the marked sensitivity to toxoid shown by these animals, they are usually Schick-positive, and their serum may contain no detectable antitoxin (that is, $<.001$ unit/ml.). Presumably the horse antitoxin has been effectively eliminated after the formation of antihorse globulin antibody.

Since horse antitoxin injected shortly after infection with *Corynebacterium diphtheriae* appeared to block active antitoxin production by the guinea pigs themselves, further experiments were carried out in which the animals were infected with toxigenic diphtheria bacilli suspended in horse antitoxin, and in which antitoxin was given prior to infection. It was found that even when relatively large doses of antitoxin were given several hours *before* infection, guinea pigs still developed pronounced hypersensitivity to diphtheria toxoid. Under these conditions, of course, toxin formed *in vivo* was immediately neutralized, and no visible necrosis occurred at the site of inoculation. Once again, the sensitized animals showed no evidence of having formed their own antitoxin.

Induction of hypersensitivity with specific precipitates. The above experiments suggested that the delayed hypersensitive state might be induced in guinea pigs by injecting minute amounts of toxoid in combination with excess antitoxin. Accordingly, specific precipitates were prepared by the addition to a rabbit antitoxin of one half that amount of purified toxoid required to bring the system to equivalence. The washed specific precipitate, composed of about 4 to 5 molecules antitoxin per toxoid molecule, was suspended in Arlcel A-Bayol F mixture containing 1 mg. ml. killed *Mycobacterium butyricum**. Six guinea pigs were each injected, in the foot pads, with an amount of suspension containing 0.8 Lf. (2.5 μ g.) "bound" toxoid. The total volume injected was 0.4 ml. per guinea pig. Six control animals were injected with 2.5 μ g. "free" toxoid suspended in the same adjuvant containing *Mycobacteria*. Two weeks later, when all of the animals were skin-tested with dilutions of purified toxoid, they showed delayed reactions even to very small doses of toxoid (TABLE 1). The character of the lesions in the 2 groups of animals differed, however. Thus, reactions appeared earlier, that is, at 2 hours, in the control group, as compared with 5 hours in the guinea pigs sensitized with specific precipitates. Moreover, with the larger challenge doses of toxoid the control animals showed skin reactions with a central hemorrhagic and necrotic area not seen in the experimental group. No antitoxin (that is, $<.001$ unit/ml. serum) could be detected in any of the animals sensitized with the specific precipitates, and no symptoms of anaphylaxis could be elicited by intravenous injection of up to 2.4 mg. purified toxoid. Although these animals produced no detectable antitoxin, they presumably formed appreciable antibody to rabbit globulin since, of 5 similarly sensitized guinea pigs which subsequently received 1 mg. rabbit gamma globulin intravenously, all died in anaphylactic shock. On the other hand, all of the animals sensitized with "free" toxoid were Schick-negative. Their serum contained 0.03 $>.16$ units antitoxin, and all showed moderate to severe symptoms of anaphylaxis following intravenous injection of 0.3 to 2.4 mg. toxoid. In 3 of the 6 animals shock was fatal within 3 minutes (TABLE 1).

* Kindly supplied by Aaron Lane, Difco Laboratories Inc., Detroit, Mich.

TABLE 1
REACTIONS IN GUINEA PIGS SENSITIZED TO TOXOID
(ALL TESTS 16 TO 18 DAYS AFTER SENSITIZATION)

Group I: 2.5 μ g. toxoid precipitated with excess rabbit antitoxin. Washed precipitate suspended in Arlaccel Bayol F with *Mycobacteria* and injected into foot pads.
Group II: 2.5 μ g. toxoid in Arlaccel Bayol F with *Mycobacteria* injected into foot pads.

No.	Skin reactions in mm. read 18-24 hrs. to challenge dose purified toxoid				Serum antitoxin (units/ml.)	Intravenous challenge	
	3	0.3	0.03	0.003 μ g.		Toxoid (μ g.)	Anaphylactic symptoms
Group I							
1	25 \times 20	13 \times 11	6 \times 6	\pm	<0.001	600	none
2	15 \times 15	13 \times 12	10 \times 10	5 \times 5	<0.001	600	none
3	20 \times 18	12 \times 11	5 \times 5	0	<0.001	600	none
4	40 \times 35	20 \times 17	10 \times 12	10 \times 9	<0.001	not done	
5	20 \times 18	12 \times 11	8 \times 7	7 \times 7	<0.001	300	none
6	25 \times 20	17 \times 16	10 \times 12	8 \times 6	<0.001	2400	none
Group II							
1	18 \times 18	10 \times 12	7 \times 8	5 \times 6	>0.16	300	severe
2	22 \times 20	11 \times 12	11 \times 8	4 \times 4	0.03	600	moderate
3	32 \times 22	15 \times 12	17 \times 13	12 \times 10	0.12	600	death
4	20 \times 18	14 \times 16	12 \times 9	10 \times 8	0.06	600	severe
5	23 \times 18	18 \times 15	11 \times 10	7 \times 7	0.03	2400	death
6	35 \times 30	—	—	—	>0.16	900	death

Effect of Mycobacteria on sensitization. The experiment just described clearly shows that a bacterial protein in amounts comparable with the small amounts that might be produced *in vivo* during infection can, if combined with excess antibody, induce the delayed hypersensitive state without formation of detectable antibody. In this case, however, the control group injected with "free" toxoid and *Mycobacteria* also showed delayed inflammatory reactions, the character of which seemed to be modified by the presence of circulating antibody. It was therefore decided to investigate the role of killed *Mycobacteria* in inducing the sensitization.

After injection of washed specific precipitates, formed in excess rabbit antibody and suspended in oil emulsion *without Mycobacteria*, guinea pigs became highly sensitive to toxoid or to crystalline egg albumin without the formation of detectable antibody. As in the preceding experiment, antibody to rabbit globulin was formed. This was evidenced by Arthus-type skin reactions following intracutaneous injection and fatal anaphylactic shock following intravenous injection of rabbit globulin. When *Mycobacteria* were omitted from the adjuvant, the antibody response of the *control* animals was unimpaired, but skin reactions seemed to be predominantly of the Arthus type, since they appeared early, reaching maximal intensity in about 4 hours. At 18 to 24 hours the skin reactions were diminishing in intensity. Attempts to transfer hypersensitivity to normal guinea pigs, using lymph node cells from animals sensitized with "free" toxoid or egg albumin in oil emulsions that did *not* contain *Mycobacteria*, were unsuccessful. On the other hand, cell transfers were successful when lymph-node cells were used from animals sensitized with specific precipitates.

Effect of species differences in antibody. Toxoid-containing specific precipitates were prepared, using antitoxin from rabbit, horse, man, and guinea pig. An excess of antitoxin from any one of these species, bound to 1 to 2.5 μ g. purified toxoid, suspended in oil, and injected into the foot pads of guinea pigs, proved equally effective in producing a high degree of hypersensitivity. More than 50 animals rendered highly sensitive by injection of specific precipitates have now been tested. In only 1 animal could as much as 0.001 unit/ml. (that is, about 0.0025 μ g. antitoxin N/ml.) be detected in serum withdrawn 2 to 3 weeks after sensitization. Finally, the following experiment demonstrates that antigen does not need to be injected in the form of a specific precipitate in order to induce the hypersensitive state. Three guinea pigs received 1000 units horse antitoxin and 3 received 70 units guinea pig antitoxin intraperitoneally. Twenty-four hours later the 6 animals were injected with 2.5 μ g. "free" toxoid in oil into the foot pads. All of the animals proved to be highly sensitive when skin-tested 3 weeks later. Only the 3 animals which received homologous antitoxin were Schick-negative at the time of skin testing. In the latter case, delayed-type sensitivity was demonstrated by cell transfer.

Conclusions

(1) Guinea pigs infected intradermally with toxigenic diphtheria bacilli develop delayed hypersensitivity of the "tuberculin" type specifically directed against diphtheria toxin or toxoid, even when a protective dose of horse antitoxin is given *before* infection. The infected animals do not form detectable circulating antitoxin.

(2) Guinea pigs develop delayed hypersensitivity, without detectable circulating antibody, to diphtheria toxoid or egg albumin following intradermal injections of minute amounts of specific precipitates formed with excess rabbit, horse, human, or guinea pig antibody. *Mycobacteria* are not required for inducing the sensitivity. Demonstrable antibody is formed against the heterologous globulin.

(3) Sensitivity may be transferred to normal guinea pigs by injection of lymph-node cells taken from the sensitized animals.

(4) Guinea pigs injected with the same amounts of free toxoid or egg albumin produce easily detectable circulating antibody. The skin reactions in these animals are predominantly of the Arthus type. Attempts at transfer with lymph-node cells have been unsuccessful.

(5) Guinea pigs sensitized by injection of free antigens incorporated in adjuvants containing killed *Mycobacteria* show skin reactions combining features of both Arthus and "tuberculin" types.

References

1. GELL, P. G. H. & I. T. HINDE. 1954. Observations on the histology of the Arthus reaction and its relation to other known types of skin hypersensitivity. Intern. Arch. Allergy and Appl. Immunol. **5**: 23.
2. CHASE, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. Proc. Soc. Exptl. Biol. Med. **59**: 134.

3. DIENES, L. 1929. The technique of producing tuberculin type of sensitization with egg white in tuberculous guinea pigs. *J. Immunol.* **17**: 531.
4. FREUND, J. & L. McDERMOTT. 1942. Sensitization to horse serum by means of adjuvants. *Proc. Soc. Exptl. Biol. Med.* **49**: 548.
5. RAFFEL, S. 1948. The components of the tubercle bacillus responsible for the delayed type of "infectious" allergy. *J. Infectious Diseases.* **82**: 267.
6. TREMAINE, M. M. & W. S. JETER. 1954. Passive cellular transfer of hypersensitivity to serum antigens in rabbits. *J. Immunol.* **74**: 96.

TRANSPLANTATION STUDIES IN PATIENTS WITH AGAMMAGLOBULINEMIA*

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The discovery of agammaglobulinemia in 1952¹ provided a new and clear-cut opportunity to gain insight into the nature and significance of the immune response in man. The initial studies²⁻⁷ established that patients with agammaglobulinemia have a disease featured by increased susceptibility to infection, absence of gamma globulin from the blood and tissues, and failure of antibody production in response to the most intensive antigenic stimulation. This form of agammaglobulinemia is an isolated defect in protein metabolism. Extreme hypogammaglobulinemia, or even agammaglobulinemia, however, may occur as a part of several diseases featured by more general abnormalities in protein metabolism, such as nephrosis,⁸ multiple myeloma,⁹ nutritional failure,¹⁰ and failure of general protein fabrication.¹¹⁻¹⁴

Since we believe that the isolated forms of agammaglobulinemia offer an unusual opportunity to study the nature of the immune response and its relationship to other biological phenomena in man, we shall limit this discussion to this form of gamma-globulin dysmetabolism. During the past 2 years we have had the opportunity of discovering and studying 12 cases of isolated agammaglobulinemia. Brief summaries of these cases included in TABLE 1 describe the clinical manifestations of the disease observed therein.

The purpose of this report is threefold: (1) to summarize briefly general studies carried out on patients with agammaglobulinemia that serve to define the disorder; (2) to report efforts involving various forms of transplantation in patients with agammaglobulinemia; and (3) to record briefly observations made during the course of pregnancy of an agammaglobulinemic patient and throughout the first year in the child born of the agammaglobulinemic mother. A detailed study of this "experiment of nature" will be reported elsewhere.¹⁵

General considerations of agammaglobulinemia. Although agammaglobulinemia is probably still to be considered an uncommon disease, it is by no means rare. To date, 56 cases have been reported in the literature,¹⁶ and the rate at which reports from every country are appearing in the medical journals indicates that available accounts describe only a sampling of the existing number of these patients.

Isolated agammaglobulinemia occurs in at least 3 distinct forms. Perhaps the most common variety is transient agammaglobulinemia of infancy. This self-limited form of agammaglobulinemia occurs in both sexes and does not appear to have a significant familial distribution. In a sense, this form represents a variety of the normal relationships. Normally, the newborn infant

* These studies were aided by grants from the Helen Hay Whitney Foundation, New York, N. Y.; the United States Public Health Service, Bethesda, Md.; the Minnesota Division of the American Cancer Society, St. Paul, Minn.; the Minnesota Heart Association, St. Paul, Minn.; and the American Heart Association, New York, N. Y.

TABLE 1
PATIENTS WITH AGAMMAGLOBULINEMIA—STUDIED AT
UNIVERSITY OF MINNESOTA HOSPITAL 1954-1955

Patient	Clinical summary	Gamma-globulin concentration		
		Free electrophoresis gm. %	Zone electrophoresis gm. %	Zinc turbidity units
Congenital agammaglobulinemia				
E.S., 9-yr.-old male	Repeated episodes of bacterial meningitis, 9 episodes bacterial pneumonia, otitis, urinary tract infections and sinusitis, and septicemia.	0.0	0.0	0
W.A., 8-yr.-old male	Bacterial meningitis, 15-20 episodes of pneumonia, bronchiectasis, repeated otitis, sinusitis, and septicemia.	0.0	0.0	0
T.A., 2-yr.-old male	Pneumonia two times and repeated bacterial respiratory infection.	0.0	0.0	0
B.H., 6-yr.-old male	Rheumatoid arthritis, pneumonia, septicemia, and repeated bacterial respiratory infection.	0.0	0.0	0
J.S., 2-yr.-old male	Repeated pneumonia and bacterial respiratory infections.	0.0	0.0	0.1
F.T., 2-yr.-old male	Repeated pneumonia (20 attacks), septicemia, and meningitis.	0.0	0.0	0
T.T., 1½-yr.-old male	Repeated pneumonia, septicemia, repeated otitis, sinusitis, and bacterial respiratory disease.	0.0	0.0	1.0
Acquired agammaglobulinemia				
F.H., 58-yr.-old male	Four-year history of recurrent infections, 17 attacks of pneumonia, septicemia, wound infection, and fatal hepatitis.	0.0	0.0	0
L.L., 31-yr.-old female	Eight-year history of 4 episodes of pneumococcal meningitis, 35 attacks of bacterial pneumonia (often pneumococcal), otitis media, and sinusitis.	0.0	0.0	0.2
Transient agammaglobulinemia of infancy				
L.M., 4-mo.-old male	Overwhelming fatal bacterial pneumonia and septicemia, recurrent otitis media, and recurrent U.R.I.	0.0	0.0	0.4
J.S., 6 mo.-old male	Three episodes of pneumonia between 3 and 6 months of age, septicemia, otitis media, and transient agammaglobulinemia.	Early, 0.05	0.05	0.3
		Late, 0.4	0.4	2.1
L.E., 4-mo.-old female	Three episodes of bacterial pneumonia, otitis media, and transient agammaglobulinemia.	Early 4 mo., 0.1	0.05	
		Late 8 mo., 0.5	0.5	
Normal: 1-16 years		0.6-1.15	0.7-1.3	2-13

derives antibody and gamma globulin by placental transfer from the mother. During the first 2 to 3 months of life there occurs a steady decrease in serum antibody and gamma globulin that describes, during the first few weeks of life, a logarithmic decay curve with a one-half disappearance time approximating the one-half life of gamma globulin as determined in other ways. As the baby begins to form gamma globulin (and probably antibody as well), a steady rise in gamma-globulin level occurs, and values approximating those of the adult are reached at about 2 years of age.¹⁷⁻¹⁹

In a few infants gamma-globulin formation and, presumably, antibody formation appear to be delayed, and gamma-globulin concentrations drop to extremely low levels at which they may remain for varying intervals between the second and sixth month. Spain *et al.*²⁰ have linked this form of agammaglobulinemia to the occurrence of overwhelming pneumonitis and pneumonia (the so-called crib-death syndrome) in infancy.

Of the 12 patients with agammaglobulinemia whom we have studied, 3 have suffered from this form of the disease. In each instance the infant had suffered recurrent respiratory infection during the period of agammaglobulinemia. It is probable, however, that the time of beginning formation of gamma globulin and antibodies is variable, and that many infants pass through a period of extreme hypogammaglobulinemia without untoward event. Until more extensive studies are available, the exact frequency and the clinical significance of transient agammaglobulinemia of infancy must remain obscure.

The form of agammaglobulinemia probably occurring next in frequency is the congenital disease. The anomaly in these patients appears to be an inborn error of metabolism transmitted as a sex-linked recessive trait.^{3 21-23 31} Thus far, this form has been observed only in male children. Brothers are often affected, and male offspring (cousins) from unaffected sisters have been involved. The history often reveals that male siblings of the mother and grandmother have died of infection, presumably with agammaglobulinemia, in infancy. Clinically, the disease is expressed as enhanced susceptibility to infection, usually beginning in the latter half of the first year of life. The infections that represent the clinical expression of agammaglobulinemia are usually bacterial in nature. These patients suffer from recurrent attacks of lobar pneumonia, bacterial pneumonitis, purulent meningitis, sinusitis, otitis media, and septicemia. For example, one of the children whom we studied experienced, over a 6-year period, 2 episodes of pneumococcal meningitis, 15 episodes of bacterial pneumonia, several episodes of septicemia, and innumerable instances of otitis media, pharyngitis, and purulent sinusitis. Another 7-year-old boy had suffered 3 attacks of bacterial meningitis, 7 attacks of bacterial pneumonia, septicemia on at least 3 occasions, several attacks of bacterial diarrhea, and frequent upper respiratory disease, apparently bacterial in etiology. In these cases the response of the infections to specific antibacterial therapy has been good. One of our patients, however, developed bronchiectasis as a consequence of the recurrent pulmonary disease. Thus far we have discovered and studied 7 children with the congenital form of agammaglobulinemia. All of our cases have been boys, and multiple cases have been observed in two sibships.

Occurring almost as frequently as congenital agammaglobulinemia is the adult, or so-called acquired, form of agammaglobulinemia. Characteristically these patients present a history indicating that they have been well and without inordinate trouble from infection up to a certain point in their lives. Then suddenly, just as with congenital agammaglobulinemia, life for them becomes a continuous round of severe, life-threatening bacterial infections. For example, we have studied two such cases: one a 30-year-old female and the other a 58-year-old male with acquired agammaglobulinemia. The former was well and had no increased frequency of infection until she was 22 years old. During the subsequent 8 years she suffered from pneumonia on 35 separate occasions, pneumococcal meningitis 4 times, septicemia on several occasions, and has almost continuous bacterial infection of the paranasal sinuses and middle ear.

The male patient was well until he was 54 years old. Without any recognized precipitating event other than the development of a huge thymoma, he began to experience recurrent infections. Over a 4-year period he suffered at least 17 separate attacks of pneumonia. In addition, several episodes of septicemia and recurrent upper respiratory disease associated with high fever kept him constantly ill with bacterial infection. Evidence from available literature indicates that the "acquired" form of agammaglobulinemia may begin in either sex at any age. The etiology and pathogenesis are usually obscure.

Study of the serum proteins of patients with either the congenital or the acquired form of agammaglobulinemia reveals that the total proteins are low, ranging from 4.9 to 6.7 gm. per cent, usually with a markedly increased albumin-globulin ratio. Electrophoretic analysis of the serum proteins, using either free or zone methodology, reveals complete absence of the gamma-globulin peak in both acquired and congenital groups. The remainder of the electrophoretically identifiable fractions are present in normal concentration. A possible exception is the antibody containing B₂ globulin, which also may be deficient in this disease.

Although this disease is termed agammaglobulinemia on the basis of the absence of gamma globulin on the electrophoretic pattern, it is doubtful that gamma globulin is completely lacking in the blood of these patients. Using highly sensitive and specific immunochemical methods, Gitlin's studies²⁴ and those carried out in our own laboratories²⁵ show that minute amounts of gamma globulin are present in the sera of children and that slightly higher concentrations are present in the sera of adults with the acquired form of the disease. The gamma-globulin concentrations of the serum from the agammaglobulinemic patients, as revealed by an immunochemical method, are summarized in TABLE 2. It is clear that all of the so-called agammaglobulinemic patients possess detectable gamma-globulin concentrations. Although overlap exists, the adult patients suffering from the acquired form of the disease tend to have significantly higher concentrations of gamma globulin than do the children with the congenital type. On the basis of these studies it seems more precise to think of all these patients as having extreme hypogammaglobulinemia rather than complete absence of gamma globulin, as the name of the disease implies.

Studies by Gitlin,²⁴ Young, Wolfson, and Cohn,⁹ Lang *et al.*,²⁶ and Good *et al.*¹⁶ establish that both the congenital and acquired forms of agammaglobulinemia

TABLE 2
GAMMA-GLOBULIN CONCENTRATION BY IMMUNOCHEMICAL
TECHNIQUE IN AGAMMAGLOBULINEMIC PATIENTS

Patient	Form of disease	Concentration of gamma globulin mg. %*
E.S.	Congenital	11.2
W.A.	"	4.2
T.A.	"	13.6
B.H.	"	10
J.S.	"	3
L.L.	Acquired	11.1
F.H.	"	40
M.O.	"	34.3
K.N.	"	97
Normal.		700-1300 mg. %

* Mean value of multiple determinations.

are due to failure of formation of gamma globulin rather than to increased loss or inordinately rapid destruction of this protein constituent. For example, in 3 of our cases where the survival of gamma globulin has been estimated by zinc turbidity, electrophoresis, and immunochemical technique, the half life of this compound has varied between 28 and 34 days. This value would indicate that the survival of gamma globulin in these patients is comparable to its survival in normal adults as revealed by the half life of gamma globulin tagged with certain radioactive isotopes.²⁷

Definition of the immunological handicap. Studies in numerous laboratories,^{1, 9, 26} including our own,^{5, 21, 25} have established the nature of the immunological handicap in these patients. Our own observations have been fivefold and may be summarized as follows:

(1) *Failure of response to ubiquitous antigens.* Both children and adults with agammaglobulinemia fail to show evidence of immune response to ubiquitous antigens. For example, all 9 of the agammaglobulinemia cases were Schick-positive at the time of study; 6 of 6 studied were Dick-test positive; none possessed measurable amounts of antibody to the streptococcal antigens (streptolysin O, hyaluronidase, streptokinase, and streptococcal ribonuclease); antibodies against herpes were uniformly absent from the serum, as were cold agglutinins, heterophile antibodies, and antibodies against all 3 types of polio virus. These findings were sharply at variance with those made on normal children and adults in our community, most of whom showed evidence of a demonstrable immune response to at least several, if not all, of these antigens.

(2) *Failure of immune response to potent bacterial antigens.* The agammaglobulinemic patients failed to respond to primary, secondary, and tertiary stimulation with potent bacterial antigens. Injections of typhoid-paratyphoid vaccine, employing intradermal, subcutaneous, and intravenous routes, on at least 5 occasions in each of our patients failed to induce antibody production. Similarly, attempts to immunize with diphtheria, pertussis, and tetanus antigens, pneumococcal polysaccharides, pneumococcal infection, and *Brucella* polysaccharide antigens resulted in no demonstrable immune response.

(3) *Absence of so-called natural antibodies from the circulation.* The so-called natural antibodies (isoagglutinins against heterologous blood groups) were completely absent from the serum in 6 of 9 cases studied. These antibodies were present in only very low titer in the other 3. Thus, 1 child was of blood group B and possessed no antibodies to A cells; 5 patients were blood group O and possessed no agglutinins against either A or B cells; and 3 patients were of blood group A and possessed only very low agglutinin titers against B cells. In no instance did these titers overlap the lowest antibody titer observed in a large group of normal and hospitalized children and adults employed as controls.

(4) *Failure of immune response and absence of toxic reactions to the intravenous or intramuscular injection of mismatched blood.* Five agammaglobulinemic patients of blood group O who possessed no isoagglutinins against A or B cells were injected intravenously with 10 to 20 cc. of mismatched blood cells. No reactions or alterations in circulating white-blood-cell concentrations were produced, and no antibodies against the mismatched cells appeared in the serum during the 20 days subsequent to the injection. In contradistinction, when immunologically normal persons with normal isoagglutinin titers were injected rapidly with 2 cc. of packed mismatched cells, within an hour they developed slight fever, sudden decrease in concentration of circulating granulocytes, and a subsequent vigorous immune response to the mismatched cells. Subsequent intramuscular, intradermal, and subcutaneous injections of mismatched cells in the agammaglobulinemic patients failed to induce the formation of isoagglutinins against the cells of the blood group injected.

(5) *Failure of immune response to potent virus antigens.* A striking clinical paradox in agammaglobulinemic patients has been the observation that their disease consists primarily of *bacterial* infections,^{1, 3, 28} and that many virus infections seem to be dealt with as in normal persons. For example, these patients may have measles, chickenpox, and vaccinia infections, express them in the usual way, recover from them, and even appear to resist their recurrence. Further, virus respiratory infections do not seem to pose any unusual problem to patients with agammaglobulinemia. It seemed possible that antibody formation against virus antigens may be intact in these patients. This possibility seems compatible with the observation that an accelerated response to vaccinia occurred upon revaccination of some of the agammaglobulinemic patients.^{3, 16} In order to test this hypothesis we have attempted to induce antibody production against virus antigens in 5 agammaglobulinemic patients, to no avail. Influenza, mumps, polio, spotted fever group, Western equine encephalitis, Q fever, and typhus-fever vaccines have been repeatedly injected into these patients without producing demonstrable antibody response. The types of antibodies sought in these experiments have included neutralization, complement-fixation, and those capable of preventing hemagglutination of red blood cells by virus particles. Except for one patient, a 58-year-old adult male with acquired agammaglobulinemia who produced a minimal 1:4 titer against type 1 poliomyelitis virus after the third inoculation, none of the agammaglobulinemic patients produced measurable antibody against any of the virus antigens employed. In one agammaglobulinemic child, poliomyelitis vaccine has been injected on at least 5 separate occasions without producing a de-

monstrable antibody titer against any of the 3 types of polio virus. From this study it seems reasonable to conclude that defense mechanisms other than the production of circulating antibody account for the relative resistance of the agammaglobulinemic patient to virus infection.

From this series of observations we conclude that most of the patients with agammaglobulinemia suffer an "immunological paralysis," that is, they are immunologically unresponsive. In some, particularly those with acquired agammaglobulinemia, the paralysis is incomplete and may better be described as a severe immunological handicap or "paresis." The observation that patients with acquired agammaglobulinemia tend to have somewhat more gamma globulin in their sera than do patients with congenital agammaglobulinemia is compatible with these findings.

Children with transient agammaglobulinemia of infancy may show a poor immunological response or they may show a very good immunological capacity, depending on whether they are being studied prior to assumption of immunological responsibility or shortly after they have become capable of gamma globulin and antibody formation. The 2 patients with transient hypogammaglobulinemia of infancy whose immunological capacity we have evaluated showed vigorous immune response to both virus and bacterial antigens at the time of our study.

Hypersensitivity reactions in the agammaglobulinemic patient. Most studies thus far reported have emphasized that the tuberculin reaction has been negative in patients with agammaglobulinemia. The tuberculin reaction was recorded as being negative in all 12 agammaglobulinemic patients studied in our laboratory. Observations on a 6-year-old male child, however, provoked consideration of the possibility that agammaglobulinemic patients might possess bacterial-type hypersensitivity even though they were unable to produce circulating antibody.^{5, 21} Following the intradermal injection of 0.1 cc. old tuberculin in dilutions of 1:1000, 1:100, and 1:10, this child developed erythema lasting 72 hours. He also reacted to second-strength purified protein derivative with an erythematous reaction lasting 72 to 96 hours. The absence of clearly definable induration made it hazardous to record this as a positive tuberculin reaction. To gain insight into the relationship between antibody production and occurrence of bacterial-type hypersensitivity, a form of delayed hypersensitivity occurring more frequently in the population at large was studied. Skin reactions of the agammaglobulinemic patients to streptokinase-streptodornase (SK-SD) (Lederle), whole group A streptococcal vaccine*, and pneumococcal vaccine† were studied. The results of the studies with the 3 vaccines were essentially similar: the agammaglobulinemic patients did not react, whereas an appreciable percentage of the population at large showed a delayed skin sensitivity to these vaccines. The results of the study carried out with SK-SD are summarized in TABLE 3. None of 7 agammaglobulinemic patients tested showed a positive skin reaction to SK-SD, while the majority of hospitalized adults and children and normal adults showed the strongly positive reactions characteristic of delayed-type hypersensitivity. We interpreted these observa-

* Prepared in our own laboratory from 8 different types of group A streptococci

† Prepared from whole organisms from a rough strain of pneumococci.

TABLE 3
BACTERIAL-TYPE HYPERSENSITIVITY TO STREPTOCOCCAL
PRODUCTS IN AGAMMAGLOBULINEMIC PATIENTS

Vaccine injected intradermally	Group	Number	No. positive	No. negative	Per cent positive
Streptokinase 10 units	Agammaglobulinemia	8	0	8	0
Streptodornase 2.5 units in 0.1 cc. pyrogen-free physiological saline	Hospitalized children 2-5 yrs.	34	16	18	47
	Hospitalized children 2-16 yrs.	44	40	4	90.9
	Healthy adults	26	22	4	85

tions to mean that agammaglobulinemic patients are deficient in both ability to develop bacterial-type hypersensitivity and in ability to form circulating antibody. However, the observations of Zinneman²⁹ and Seltzer,³⁰ who discovered agammaglobulinemic patients sensitive respectively to tuberculin and histoplasmin, and of Kulneff *et al.*³¹ and Porter,²³ who observed the development of tuberculin sensitivity in agammaglobulinemic patients vaccinated with BCG, made reconsideration of this conclusion essential. Experiments bearing directly on this point are described later in this paper.

Hematological studies in agammaglobulinemia. Because it had been observed^{32, 33, 34} that agammaglobulinemia may be associated with lymphopenia, and because we have long been interested in the cytological basis of the immune response,³⁵⁻⁴¹ we carried out a systematic study of the blood and blood-forming tissues in 12 cases of agammaglobulinemia. The findings, reported elsewhere,^{5, 21, 22, 42, 43} were of interest. It was found that among our cases hematological disturbances were the rule. Maturational and developmental defects occurred in the cells of the neutrophil, eosinophil, and lymphocytic series. For example, among the children with the congenital form of the disease, particularly during the early years of life, episodic profound neutropenia was common. At first these episodes were attributed to the adverse hematologic effects of overwhelming infections that occurred so frequently in these children, but, on several occasions after our hematological study was initiated, we observed that neutropenia occurred as an isolated event in the complete absence of demonstrable infection. One of the children developed a profound, persistent neutropenia, and another, an apparent cyclic neutropenia. Serial study of the bone marrow during the neutropenic phase revealed evidence of marked shift to the left among the neutrophilic precursors and apparent arrest in their maturation. In a 58-year-old man, extreme eosinopenia, also of an aregenerative type, was observed. As already mentioned, others had observed persistent lymphopenia^{33, 34} as a concomitant of this disease. The deficiency of each of these circulating elements of the peripheral blood appears to be attributable to failure of the formation of the definitive cells in the hematopoietic tissues and does not seem to be associated with excessive destruction or loss of the cellular element involved. Additional hematologic abnormalities were observed. One of the patients with acquired agammaglobulinemia

apparently developed his clinical disease in temporal association with the development of a huge thymoma. In the other 30-year-old female patient with acquired agammaglobulinemia, an ill-defined but definite disease of the hematopoietic tissues was observed. Early in her disease this patient was found to have generalized lymphadenopathy and hepatosplenomegaly associated with the occurrence of a Coombs-test-negative hemolytic anemia, neutropenia, and thrombopenia. The anomalies of the peripheral blood appeared to be a function of hypersplenism, a conclusion supported by the observation that these manifestations were relieved by splenectomy. Microscopic study of the spleen, lymph nodes, and bone marrow of this patient, however, revealed excessive proliferation of the stromal (reticulum mesenchymal) cells in this instance.

In addition to these diverse and apparently unrelated hematological abnormalities occurring together with agammaglobulinemia, lymph-node and bone-marrow biopsies reveal more consistent and perhaps more interesting anomalies.

In the patients with congenital agammaglobulinemia, the lymph nodes tend to be small, possessing a relatively thin cortex, poorly developed lymphoid follicles, and a relatively acellular medullary portion. In our patients, as in most reported cases, the peripheral blood and bone marrow contained at least normal numbers of lymphocytes. The bone marrow and lymph nodes of all of the patients with agammaglobulinemia were devoid of plasma cells, however, and could be distinguished from normal or other pathological nodes and marrow by this criterion. Even more striking was the observation that the differentiation and proliferation of plasma cells that normally follow antigenic stimulation in immunologically normal persons failed to occur in the agammaglobulinemic patients. Even secondary and tertiary antigenic stimulation regularly productive of marked plasma-cell and pyroninophilic-cell response in the bone marrow and regional lymph nodes draining the site of antigenic stimulation produced no plasma-cell or pyroninophilic-cell response in patients with agammaglobulinemia. Furthermore, chronic inflammatory exudate, that is, areas of bronchiectasis that were featured by plasmacytosis in the immunologically normal patient, contained no plasma cells in the agammaglobulinemic patient. Another abnormality of the lymph nodes of the agammaglobulinemic patient was characteristic. Whereas the lymph nodes of immunologically normal persons regularly developed clearly defined secondary follicles following antigenic stimulation, such follicles were completely absent from the nodes of patients with agammaglobulinemia. These observations, taken together with other studies of the immune response⁴¹⁻⁴⁹ and gamma-globulin dysmetabolism⁵⁰ in man and experimental animals, lure one to attempt to unify the diverse expressions of hematological disease in agammaglobulinemia and explain the protein dysmetabolism and immunological deficit on the same cellular basis.

Could it be, for example, that the fundamental disease in these patients resides in the fact that multipotent mesenchymal cells (reticulum of Maximow) are reflected in diverse ways in different patients? In some, the defect might

be expressed as transient, intermittent, or persistent failure of heteroplastic maturation of neutrophils, resulting in transient, persistent, or cyclic neutropenia. In others, failure of heteroplastic metamorphosis to eosinophils or lymphocytes might occur on a similar basis. In still others a benign or even malignant⁵¹ proliferative abnormality among these same mesenchymal cells might underly or reflect their basic functional disturbance. This inadequacy of the hematopoietic reticulum (mesenchymal cells) could then gain uniform expression in the failure of heteroplastic development of these cells along the plasma-cell line in response to antigenic stimulation, and regular failure of antibody and gamma globulin production would be the concomitant.

If this pathogenic concept is correct, one might predict the ultimate discovery of agammaglobulinemia associated with hypoplastic or aplastic anemia, since the red-blood-cell series is another cell line continually derived from the multipotent elements. A recent case discovered by Loeb,⁵² in which agammaglobulinemia was indeed associated with areregenerative anemia, neutropenia, and thymoma seems to fit this prediction.

Certain it is that, whatever its basis, the agammaglobulinemic patient, unlike the normal person, appears to be unable to respond to antigenic stimulation by the production of plasma cells, antibody, and gamma globulin. In FIGURE 1 lymph nodes from a normal child and a patient with agammaglobulinemia are compared. Both nodes were taken from the inguinal region 4 days following injection of antigen into the anterior medial aspect of the thigh. On FIGURES 2a and 2b sections of these nodes are illustrated; on FIGURE 3 imprints of them are shown. Plasma-cell development is abundant in the node from the normal child and completely absent from that of the patient with agammaglobulinemia.

Many other studies attempting to gain insight into the nature of the defect in protein synthesis have been carried out in patients with agammaglobulinemia. These have included:

(1) Extensive study of liver function, which has proved to be normal in every way.⁵³

(2) Similar extensive study of adrenal function, which is likewise normal.

(3) Study of the concentrations of other proteins, as revealed by electrophoretic and chemical analysis. These have all fallen within normal range.²²

(4) Detailed evaluation of the coagulation mechanism, which has proved to be without fault in these patients.⁵⁴

(5) Study of response to gram-negative bacterial endotoxins. Skin reactions, chills, malaise, fever, and the rapid development of refractoriness occur, and differ in no way in agammaglobulinemic and normal persons.

(6) Study of the acute-phase reactions in patients with agammaglobulinemia. These show no deviation from the normal. For example, C-reactive protein appears in the blood during acute disease and, following appropriate noxious stimuli and the expected changes in sedimentation rate, serum mucoprotein concentration, fibrinogen, heparin precipitable protein, and alpha globulin occur during acute disease, just as in normal persons.

(7) Complement concentrations measured by the method of Wedgewood and Janeway⁵⁵ were found to be normal or slightly high in these patients.

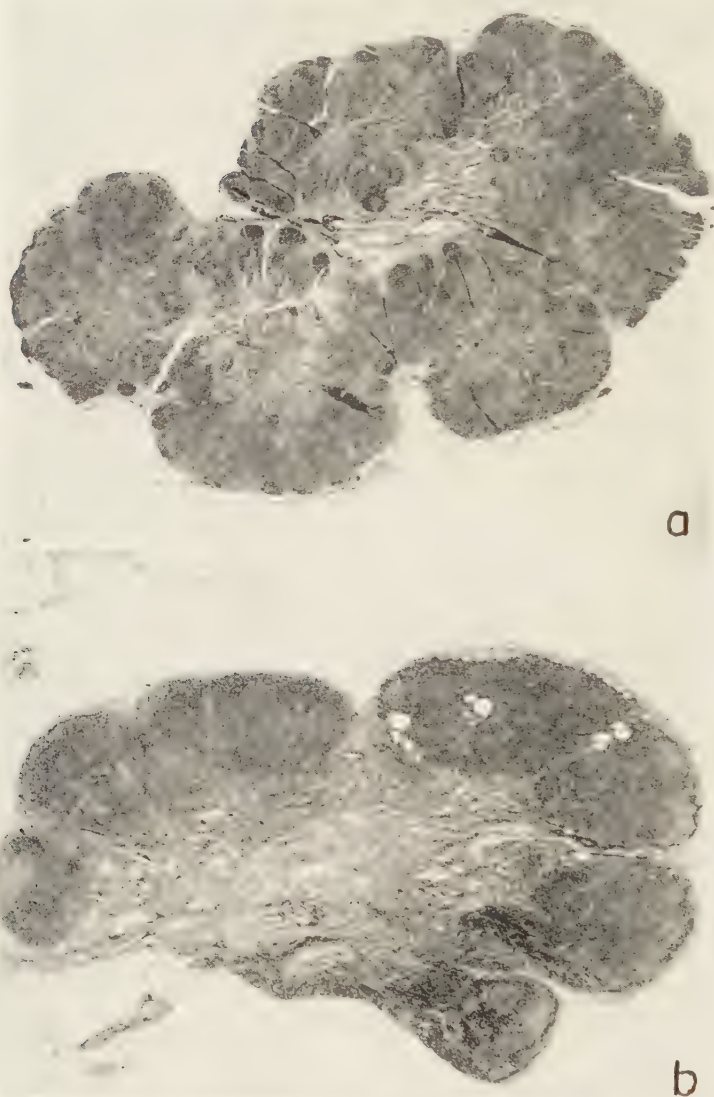


FIGURE 1. Comparison of the lymph nodes from an immunologically normal person and an agammaglobulinemic child following primary antigenic stimulation. The lymph node shown in (a) was taken from the right groin 4 days after the injection of 1.0 cc. TAB bacterial antigen in the right thigh of a normal child. The relative thickness of the cortex and the numerous well-developed primary and secondary follicles stand in contrast to those observed in lymph node (b), which was removed from the right groin of an agammaglobulinemic child 4 days following identical antigenic stimulation. Note the relative acellularity of the medullary portion of the node from the agammaglobulinemic child.

(8) Preliminary studies carried out by Pillemer on blood samples taken from our agammaglobulinemic patients have indicated that in most of these patients at least normal concentrations of properdin are present in the sera.

Transplantation studies in agammaglobulinemic patients. With these observa-

tions as background, we decided that the agammaglobulinemic patient might provide a unique opportunity to study the phenomenon of transplantation in man and might be an ideal subject in whom to carry out inquiry into the basis of transplantation failure. These investigations were undertaken with full realization of the lack of uniformity of the human host and resignation to the inevitability that the numbers of subjects in the experimental groups would be small. Our previous studies had further established that biologic variation within the group of agammaglobulinemic patients might be considerable and could present a problem in interpretation. The driving force behind these investigations was twofold:

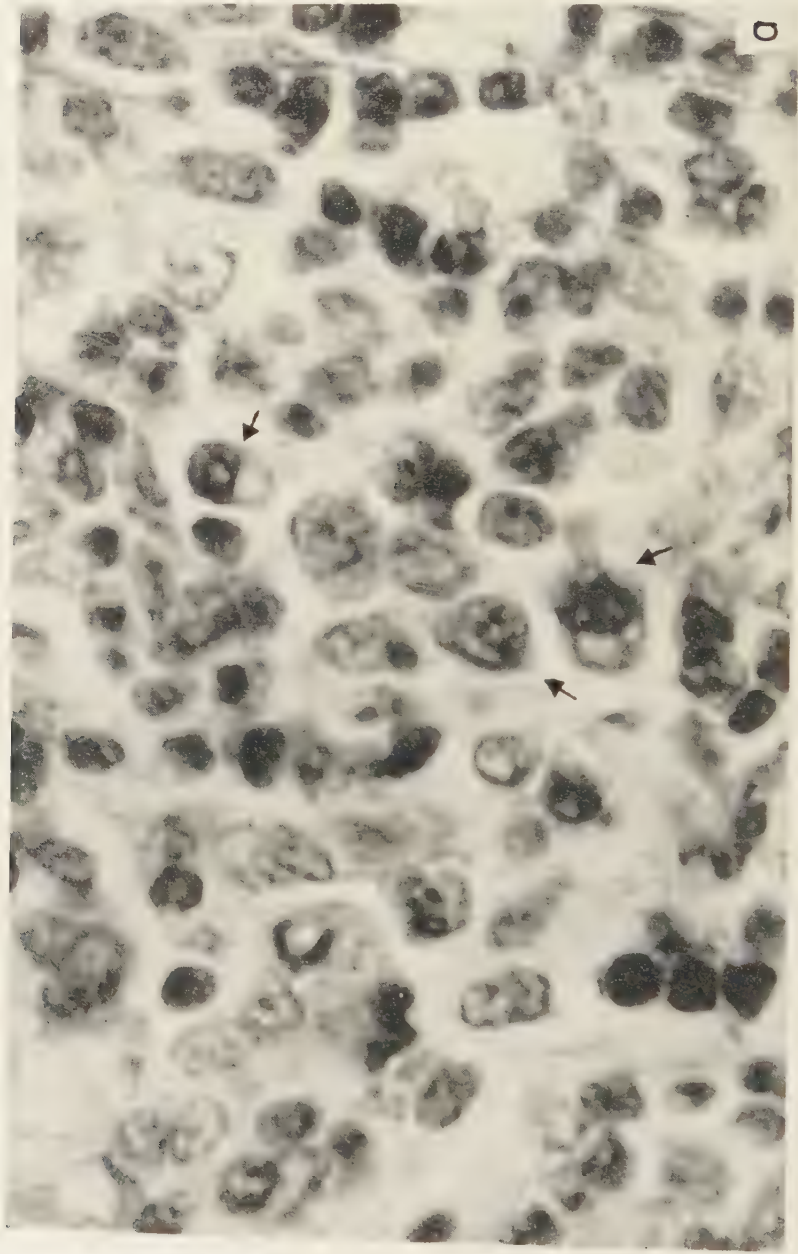
(1) The availability of the "experiment of nature"¹⁵⁶ represented by these cases seemed to offer a unique opportunity to gain further insight into the nature of the immune response, the basis of transplantation failure, the relationship of the various types of hypersensitivity to one another, and the relationship of immune phenomena to protein synthesis in man.

(2) It seemed possible that these patients, plagued as they have been by almost continuous infection, might indeed benefit from transplantation of immunologically active tissues in an effort to reconstitute their immunological capacity.

The investigations carried out thus far may be described as follows: (1) attempts to alter antibody and gamma-globulin synthesis in normal subjects by injection of plasma or leukocytes from agammaglobulinemic patients; (2) studies of the effect of normal blood and gamma globulin on the immunological capacity of agammaglobulinemic subjects; (3) studies of the effect of administration of normal leukocytes on the capacity of patients with agammaglobulinemia to develop delayed (bacterial-type) hypersensitivity and to participate in the immune response; (4) attempts to produce the development of bacterial type hypersensitivity, *de novo*, in agammaglobulinemic subjects and to transfer this type of reactivity to normal persons by injection of leukocytes and serum from sensitized agammaglobulinemic subjects; (5) studies of the capacity of agammaglobulinemic patients to reject skin homotransplants; (6) studies of the transplantation of lymph nodes to agammaglobulinemic patients and the response to antigenic stimulation following lymph-node transplantation; (7) studies of the effect of a transplant of nature, "pregnancy" on the gamma globulin level, and antibody production in an agammaglobulinemic female; and (8) studies of the immune response and gamma-globulin production during the first year of life in a baby born of an agammaglobulinemic mother.

Since each of these investigations appears to have revealed something provocative to those concerned with transplantation in man, these studies will be briefly described in the order in which they were performed.

Homotransplantation of skin to patients with agammaglobulinemia. Since extensive studies carried out primarily on experimental animals appeared to indicate that transplantation failure has an immunological basis, it was considered possible that patients lacking in immunological capacity might be able to accept skin transplants. The availability of patients with agammaglobulinemia provided an opportunity to test this hypothesis. Thus far transplantation of skin from normal to agammaglobulinemic patients has been carried out in 5



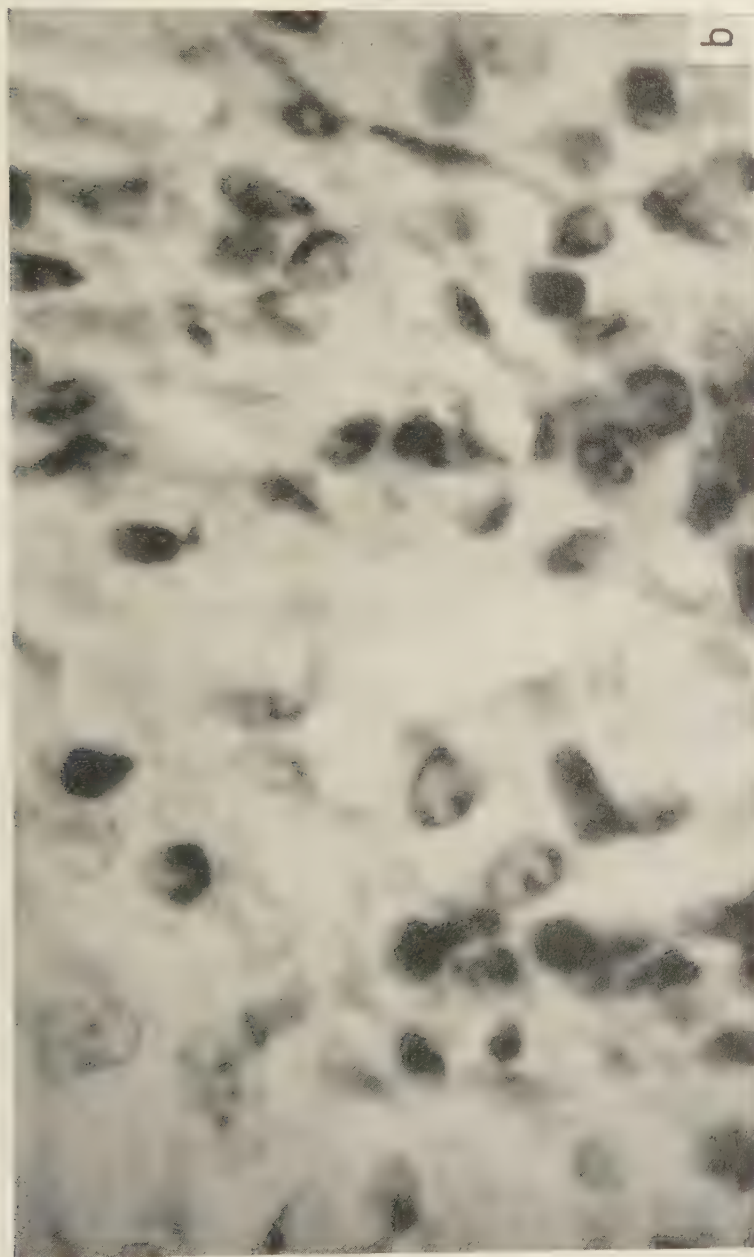


FIGURE 2. Comparison of the medullary portion of the lymph node from (a) a normal child 4 days following antigenic stimulation and from (b) an agammaglobulinemic child 4 days following comparable stimulation with TAB vaccine. Note the abundance of plasma cells in the lymph node of the normal child following antigenic stimulation and their absence from the medullary portion of the nodes from the agammaglobulinemic child. $\times 1000$.

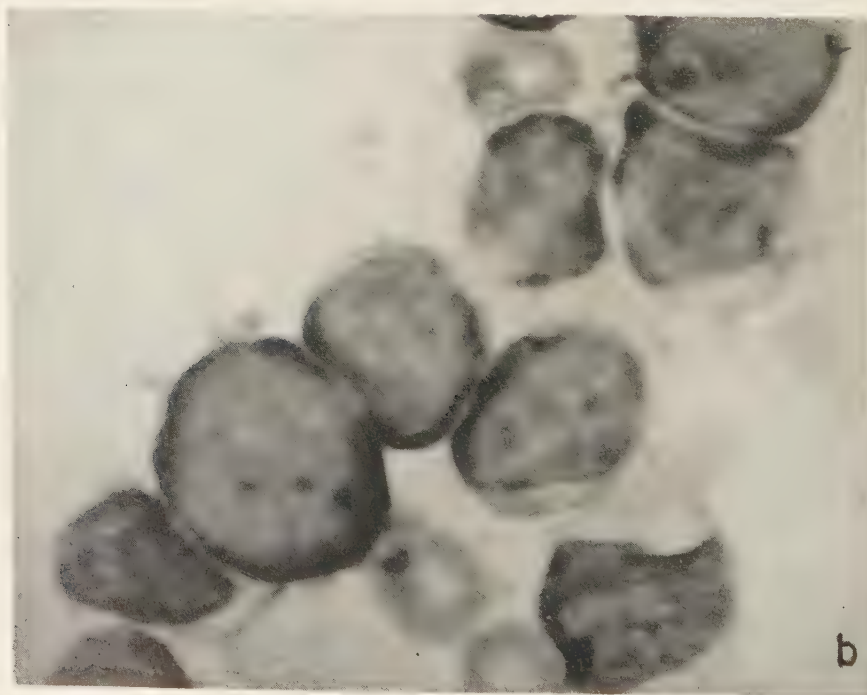
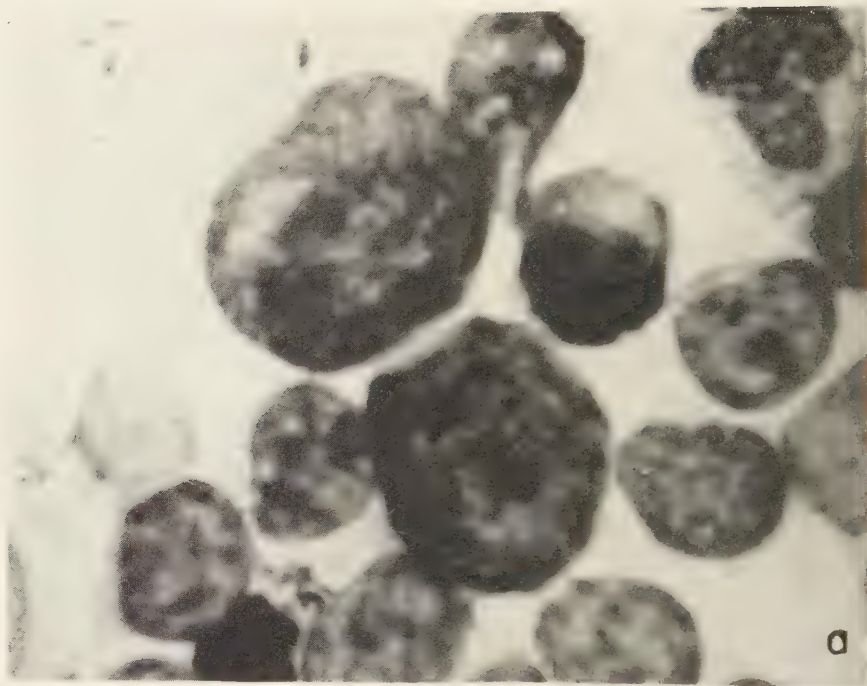


FIGURE 3. Comparison of imprints from (a) the nodes of the normal child and from (b) the agammaglobulinemic child 4 days following antigenic stimulation with TAB vaccine. Note the plasma cells in the imprint of the node of the normal child and their absence from the node of the agammaglobulinemic patient.

TABLE 4
HOMOTRANSPLANTATION OF SKIN FROM NORMAL ADULTS TO
CHILDREN WITH CONGENITAL AGAMMAGLOBULINEMIA

Patient	Source of skin	Type of graft	Result
E.S., 7-year-old male with congenital agammaglobuli- nemia Blood group O	45 year-old female Blood group A	Split thickness 4 × 3.5 cm. Full thickness 4 × 3.5 cm.	100% initial take 100% survival 23½ mo.* 100% initial take* 100% survival 23½ mo.†
W.A., 6-year-old male with congenital agammaglobuli- nemia Blood group O	31 year-old male Blood group A	Split thickness 4 × 4 cm. Full thickness 4 × 4 cm.	100% initial take 100% survival at 14 mo.* 80% initial take* 100% survival at 14 mo.

* Grafts have appeared to grow with the size of the leg.

† Preliminary sexing of the skin indicates that the graft possesses epithelial cells 37 per cent of which have female sex chromatin markings. J. L. Emery and M. McMillan, 1954, *J. Pathol. & Bacteriol.*, vol 68, p. 17).

instances. In 2 cases, skin has been transplanted from normal adults to 2 children with congenital agammaglobulinemia in whom prior studies had established existence of complete immunological unresponsiveness to a wide variety of antigens. As indicated on TABLE 4, both full-thickness and split-thickness skin homografts were applied to the subcutaneous tissue of agammaglobulinemic patients. The grafts were placed on an area freshly exposed by the removal of full-thickness skin from the agammaglobulinemic patient. The skin removed was in turn transplanted to a suitable immunologically normal recipient. As shown in TABLE 4, the pieces of skin transplanted in each instance were of relatively large size. In one of the cases the transplanted skin was successfully tattooed with India ink prior to transplantation. Whereas the skin of the agammaglobulinemic children transplanted to the immunologically normal recipients at first "took," then became necrotic and sloughed in the usual time (3 to 4 weeks), the transplants on the agammaglobulinemic patients showed no evidence of reaction, quickly regained the appearance of normal skin, and survived for prolonged periods.^{57, 58} At the present writing, both the full- and split-thickness grafts transplanted from a 45-year-old female to an unrelated 7-year-old agammaglobulinemic male have remained in place 23½ months. The unchanged tattoo marking of the full-thickness graft is strong support for the concept that the homologous skin transplanted to the child remains in place and probably represents a permanent "take." The skin graft placed on the thigh of the second agammaglobulinemic child, following a 90 per cent initial take, has remained in place 14 months following transplantation. Illustrated in FIGURES 4 and 5 are the successful homotransplants; in FIGURE 4 at 6 months and in FIGURE 5 at 13 months after transplantation in case 1. FIGURE 6 illustrates the case 2 transplant 14 months after application.

Additional skin transplants were attempted in 2 adults with acquired agammaglobulinemia, and in 1 child suffering from the transient agammaglobulinemia of infancy mentioned above. In 1 of the adults, the transplant failed



FIGURE 4. Illustration of a successful homotransplant in a child with congenital agammaglobulinemia. This figure shows the graft 6 months after transplantation. The proximal split thickness and the distal-tattooed full thickness may be seen.

to take initially and, when observed 5 days after application, had already been dislodged from its bed in the recipient skin. This full-thickness transplant was considered to be an initial failure and will not be further discussed.

In the second adult with acquired agammaglobulinemia, the experimental result was most interesting. Prior studies had established that this patient, although having a profound immunological handicap, possessed minimal capacity for immunological response. The latter was indicated by a slight response of isoagglutinin titer after injection of mismatched cells and a minimal response to type 1 polio virus after the third immunizing injection. Transplantation of full-thickness skin from this patient to an immunologically normal patient resulted in an initial "take," followed in 14 days by beginning rejection and, 10 days later, by complete slough. In contradistinction, a full-thickness homograft to the denuded area on the thigh of this patient took at first, looked healthy for from 9 to 10 weeks and then, between the 10th and 16th weeks, was gradually destroyed.

Finally, a split- and full-thickness homograft was placed on the thigh of a child with transient agammaglobulinemia of infancy. Although this child's gamma-globulin concentration was less than 50 mg. per cent at the beginning



FIGURE 5. Illustration of a successful homotransplant in a child with congenital agammaglobulinemia. This figure shows the graft in FIGURE 4, 13 months after transplantation. This graft has remained in place for 23½ months at the present writing.

of the study when the child was 6 months old, it was found, in retrospect, that this concentration had increased to 200 mg. per cent at the time the skin graft was applied and had risen still further to 400 mg. per cent at the termination of the experiment. Immunological studies revealed that this patient formed antibodies against diphtheria, pertussis, typhoid-paratyphoid, and mumps antigens during the period when the graft was in place. As in the immunologically normal person, this patient's graft at first "took," became progressively necrotic beginning 14 days after application, and was finally completely sloughed 41 days after application. FIGURES 7 and 8 illustrate the reaction to the homograft and its complete rejection 41 days after the initial transplantation (FIGURE 9). The latter observation, as well as that of homograft rejection in a 58-year-old male with acquired agammaglobulinemia, must be taken as evidence that transplantation failure can occur when the level of circulating gamma globulin is far below that normally present in the serum. The results of this study are summarized in TABLE 5.

The observations on immunologically normal persons transplanted with skin derived from agammaglobulinemic patients are summarized in TABLE 6. In each instance, initial "take" of the homotransplant was excellent, necrosis



FIGURE 6. The homotransplanted skin of the thigh of case No. 2, 14 months following transplantation.

began before the end of the third week, and complete slough occurred at the latest by the 37th day. FIGURES 10 and 11 illustrate the characteristic rejection of skin homografts in the immunologically normal person.

FIGURES 12A and 12B compare the histological appearance of a skin homograft undergoing slough 18 days after application in an immunologically normal person with a biopsy taken from the transplanted skin in an agammaglobulinemic patient 13 months after its application. Note the intense mononuclear-cell infiltrate in the immunologically normal patient and complete lack of cellularity in the skin graft on the agammaglobulinemic patient. The carbon particles in the dermis are the result of the India-ink tattoo applied to the homograft prior to transplantation 13 months previously.

Evaluation of immune response following transplantation of skin in agammaglobulinemic patients. Because of the possibility that skin from an immunologically normal host might contain reticuloendothelial elements and introduce immunological capacity in the previously unresponsive agammaglobulinemic host, the following experiments were performed:

The response of the agammaglobulinemic patients to typhoid-paratyphoid, mumps, polio virus, and diphtheria antigens was tested prior to, and at varying



FIGURE 7. The characteristic transplantation failure that occurred following transplantation of skin to a child with transient agammaglobulinemia of infancy. Shown here is the initial take 14 days after application. See FIGURES 8 and 9 for the subsequent history of this graft.



FIGURE 8. The characteristic transplantation failure that occurred following transplantation of skin to a child with transient agammaglobulinemia of infancy. Shown here is the severe reaction to the graft 7 days after the time of FIGURE 7.



FIGURE 9. The characteristic transplantation failure that occurred following transplantation of skin to a child with transient agammaglobulinemia of infancy. Shown is the site of the graft illustrated in FIGURES 7 and 8, 6 weeks following the transplantation.

intervals following, skin transplantation. The 2 children in whom apparently permanent survival of skin grafts occurred were retested for immunological capacity 3 and 6 months after transplantation of skin, respectively; the 58-year-old man who showed prolonged survival of the skin graft was retested 4 weeks after its application. At no time in any of the 3 patients tested were demonstrable amounts of antibody produced.

Effect of injection of agammaglobulinemic serum and cells on antibody and gamma-globulin formation on immunologically normal recipients. Since it was considered possible that a circulating substance capable of inhibiting the immune response was present in patients with agammaglobulinemia, experiments designed to test this hypothesis were carried out. Seventy-five cc. of heparinized plasma obtained from a 9-year-old agammaglobulinemic child was injected intravenously into a 2-year-old immunologically normal child with irreversible hydrocephalus. Blood samples were taken from the recipient child prior to injection of the plasma and at weekly intervals for 1 month thereafter. Forty-eight hours following injection of the plasma the recipient child was injected subcutaneously with 0.5 cc. of both TAB vaccine (Wyeth) and DPT vaccine (Wyeth). A Schick test performed prior to injection of plasma was positive. Blood samples of the recipient were analyzed for total protein, zinc turbidity, paper and free-electrophoretic patterns, and agglutinins against

TABLE 5
TRANSPLANTATION OF SKIN FROM NORMAL ADULTS TO PATIENTS
WITH ACQUIRED AND TRANSIENT AGAMMAGLOBULINEMIA

Patient	Source of skin	Type of graft	Result
F.H., 58-year-old male with acquired agam- maglobulinemia of 4 years duration Blood group A	Adult female, blood group O	Split thickness 2.5×5 cm. Full thickness 2.5×5 cm.	Initial failure, 0% take 100% initial take, intact at 10 weeks, gradual type fail- ure between 10 and 16 weeks
L.L., 30-year-old fe- male with acquired agammaglobuli- nemia Blood group O	Adult male, blood group A	Full thickness 3×4 cm.	Initial failure, 0% survival at 5 days, no reaction in re- gional node
J.S., 7-month-old male with transient agammaglobuli- nemia of infancy Blood group O	Adult female, blood group B	Full thickness 3×2 cm.	100% initial take, be- ginning reaction 14 days after applica- tion, sloughed com- pletely 27 days later

H, O, and B antigens. Schick tests were applied before, 24 hours after, and 3 weeks following transfer of plasma. As shown in TABLE 7, the results of this study were entirely negative. Injection of agammaglobulinemic plasma had no effect on serum concentration of gamma globulin, did not prevent conversion from positive to negative Schick test, and did not interfere with the production of agglutinins by the recipient child. Indeed, the abrupt rise in antibody titer of the recipient child was equal to, or greater than, the response obtained in most normal children following comparable antigenic stimulation.

Similar studies carried out following subcutaneous injection into an immunologically normal adult of 8.4×10^8 viable leukocytes derived from the peripheral blood of an agammaglobulinemic patient indicated that blood cells of an agammaglobulinemic patient also failed to interfere with normal gamma-globulin synthesis and antibody production.

TABLE 6
HOMOTRANSPLANTATION OF SKIN FROM PATIENTS WITH AGAMMAGLOBULINEMIA
TO IMMUNOLOGICALLY NORMAL PERSONS

Patient	Source of skin	Fate of graft
J.D., 3-yr.-old F.	Congenital agamma- globulinemia	(1) 100% initial take
K.D., 5-yr.-old F.	Congenital agamma- globulinemia	(2) Complete rejection at 37 days (1) 90% initial take
M.O., 20-yr.-old M.	Acquired agammaglob- ulinemia	(2) Complete rejection at 30 days (1) 60% initial take
H.B., 33 yr.-old M.	Acquired agammaglob- ulinemia	(2) Complete slough at 24 days (1) 100% initial take (2) Complete slough at 25 days

Effect on the immune response of injecting circulating leukocytes from normal persons into agammaglobulinemic patients. In an initial effort to provide the agammaglobulinemic patient with immunological responsiveness, the following experiments were performed:

Five normal healthy adults possessing an extreme degree of skin hypersensitivity to SK-SD were immunized by intradermal and subcutaneous injection of 0.5 cc. TAB vaccine (Wyeth). Each showed vigorous immune response to this stimulation, developing agglutinating antibody titers against H, O, and B antigens ranging from 1:640 to 1:5120. Four weeks were allowed to elapse, and each donor was again injected subcutaneously with 0.5 cc. TAB vaccine. Two days thereafter each donor was bled of 500 cc. of whole blood into a siliconized bottle containing heparin, and the red cells were separated from the white cells by a modification of the technique employed by Lawrence.⁷⁷ The leukocytes from each donor were collected by gentle centrifugation (1000 r.p.m.) and resuspended in saline. The number of cells and the percentage of viability were estimated. The recipient agammaglobulinemic patients included 2 children with the congenital form of the disease and an adult with acquired agammaglobulinemia. The recipients were skin-tested with SK-SD antigen and tuberculin prior to transfer of cells and 48 hours, 3 months, 1 year, and 18 months following transfer. The effect of leukocyte transfer on the formation of antibody by the recipient agammaglobulinemic patients was studied by evaluating their response to TAB vaccine prior to leukocyte transfer, by testing for production of typhoid agglutinins during the 10-day period following injection of the leukocytes, and by studying the response to TAB vaccine injected 4, 10, and 17 days after transfer of white blood cells.

The results summarized on TABLE 8 show that bacterial-type hypersensitivity is readily transferred by injecting leukocytes from sensitized normal donors intravenously and subcutaneously into the agammaglobulinemic recipient.

In contradistinction, leukocytes (including large numbers of circulating lymphocytes) obtained from immunologically normal donors during a period of antibody production failed to produce demonstrable amounts of antibody after intravenous and subcutaneous injections in agammaglobulinemic recipients. Further antigenic stimulation, beginning 10 days following injection of the leukocytes, did not result in agglutinin production.

Effect of injection of whole blood and gamma globulin on the immune response in patients with agammaglobulinemia. The association of immunological unresponsiveness with agammaglobulinemia in these patients made it necessary to consider the relationship of gamma globulin to antibody and vice versa. Although small amounts of antibody may be found in protein fractions other than the gamma globulin,⁵⁹ the majority of the serum antibodies are contained in this protein component. Since evidence has been presented indicating that proteins related to gamma globulin may be found throughout the electrophoretic spectrum,^{60, 61} it is possible that all the antibodies are in reality "gamma globulins." If this be true, then it is not difficult to postulate that all of the gamma globulin is antibody. Were this the case, then one would anticipate that failure of production of gamma globulin would be associated with failure of synthesis of the antibody, and vice versa. This actually is true



FIGURE 10. The homograft transplantation failure that uniformly accompanied the transplantation of skin from azanmagadalinemic patients to immunologically normal persons. This figure illustrates the initial take of a full-thickness tattooed skin graft 10 days following application of the skin to the granulating surface of the knee of a normal child. See FIGURE 11 for the subsequent history of this graft.



FIGURE 11. The homotransplantation failure that uniformly accompanied the transplantation of skin from agammaglobulinemic patients to immunologically normal persons. This figure shows the necrosis and sloughing of the homotransplant shown in FIGURE 10, 4 weeks after the initial transplantation. It will be noted that subsequent autografting has been successful in this same granulating area.



FIGURE 12. Above (a) is shown a microscopic view of a biopsy taken from the graft 14 days following the transplantation of skin from an agammaglobulinemic patient to an immunologically normal person. Note the marked inflammatory process and the beginning destruction of the dermis and epidermis.
Below (b) is shown a microscopic view of a biopsy taken from the homograft on an agammaglobulinemic patient 13 months following transplantation. At no time did detectable inflammatory reaction appear about the skin graft. The site of the punch biopsy performed in the center of the graft healed with apparent epithelialization from the graft itself.

TABLE 7

EFFECT OF AGAMMAGLOBULINEMIC PLASMA ON ANTIBODY FORMATION AND GAMMA-GLOBULIN CONCENTRATION*

Procedure: 2-year-old child injected intravenously with 75 cc. agammaglobulinemic plasma, 48 hours later injected subcutaneously with 0.5 cc. TAB vaccine.

Time of serum sample	Typhoid agglutinins†			Gamma globulins	
	H	O	B	%	Total gm. %
Before agammaglobulinemic plasma.....	0	0	0	18.5	1.50
Two days following agammaglobulinemic plasma prior to TAB.....	0	0	0	15.4	1.38
Two days following TAB.....	0	0	0	20.1	1.56
Nine days following TAB.....	1/2560	1/1280	1/2560	18.3	1.1
Sixteen days following TAB.....	1/2560	1/1280	1/2560	18.3	1.3
Twenty-six days following TAB.....	1/2560	1/1280	1/2560	22.8	1.5

* Gamma globulin estimated by paper electrophoresis and total protein by biuret technique.

† Typhoid-paratyphoid immunizing vaccine (Wyeth).

in agammaglobulinemia. Several alternative explanations of this relationship exist, however. Among these is the possibility that the presence of normal gamma globulin is in some way essential to the synthesis of antibody. In this case, failure of formation of gamma globulin would result in failure of formation of antibody even though the mechanism of the latter process were intact. To test this hypothesis, as well as a corollary that lack of availability of some other component of fresh, whole blood essential to the immune response might be lacking in agammaglobulinemia, 2 sets of experiments were carried out.

TABLE 8

TRANSFER BY LEUKOCYTES OF BACTERIAL TYPE HYPERSENSITIVITY TO PATIENTS WITH AGAMMAGLOBULINEMIA

Patient	Skin reaction to SK-SD prior to injection of WBC	Skin reaction to SK-SD 48 hours after injection of WBC from sensitive donor	Skin reaction 4 months after injection of WBC	Skin reaction 1 year after injection of WBC
E.S., 7-yr.-old male with agammaglobulinemia	neg	++	++++	+++
W.A., 6-yr.-old male with congenital agammaglobulinemia	neg	++	+	neg
F.H., 58-yr.-old male with acquired agammaglobulinemia*	neg	+++	--	--
<i>Not given WBC injection</i>				
T.A., 1-yr.-old male with congenital agammaglobulinemia	neg	neg	neg	neg
L.L., 30-yr.-old female with acquired agammaglobulinemia	neg	neg	neg	neg

* Positive tuberculin reaction developed in this patient which lasted 3 months following transfer of approximately 1 billion WBC from a tuberculin positive patient.

In the first, an agammaglobulinemic child previously shown to be immunologically unresponsive was transfused with 750 cc. of whole blood. Beginning 24 hours thereafter, immunological responsiveness was tested with 4 different immunological stimuli*. No immune response occurred to any of these injections.

In the second, in 2 separate experiments, large amounts of gamma globulin, 4 cc./kg., or 0.6 gm./kg., were injected intramuscularly in each of two agammaglobulinemic patients in whom prior studies had established the immunological unresponsiveness. Three days following injection of the gamma globulin, the serum concentration of the latter had increased from 4 and 10 mg. per cent, respectively, to approximately 500 mg. per cent. Following injection of the gamma globulin, each child was given a series of 3 injections of typhoid-paratyphoid vaccine, mumps vaccine, and diphtheria toxoid. Bleedings were taken 5, 10, 15, and 25 days following the injection of the vaccines, and determination of immune response studied by appropriate serological methods. Just as had been the case prior to administration of gamma globulin, no antibody production occurred even when the immunization was attempted while the patients had serum gamma-globulin concentrations only slightly lower than normal.

These observations would lend support to the view that in patients with agammaglobulinemia the defect in synthesis involves both gamma globulin and antibody, and would seem to rule out the possibility that the defect in synthesis involves gamma globulin alone. The observations, of course, would also be in accord with the popular hypothesis⁶² that antibody formation reflects a specific end folding of the globulin molecule at a terminal stage of its formation and does not represent reconstruction or folding of the "mature" protein molecule.

Effect of exogenous gamma globulin on the formation of plasma cells in patients with agammaglobulinemia. Since plasma-cell formation had been shown to be deficient in patients with agammaglobulinemia⁴³ and since it has been postulated that plasma-cell development is secondary to and not causally related to the production of gamma globulin and antibody, it was reasoned that the parenteral administration of large amounts of gamma globulin to a patient with agammaglobulinemia, together with a study of the bone marrow and regional lymph nodes, might test this notion. Consequently, over a 2-week period, a 21-kg. agammaglobulinemic child was given 160 cc. of Red Cross gamma-globulin concentrate 8 cc. kg. in a series of 3 intramuscular and subcutaneous injections of 50, 50, and 60 cc. respectively over a 2-week period. Three days after the final injection, when the serum gamma-globulin level was at its peak concentration of 1.12 gm. per cent, bone marrow was sampled. A regional lymph node draining a site of deposit of 25 cc. of gamma globulin was then removed 4 days following the injection of gamma globulin. The results were clear-cut. No plasma-cell development occurred in either location. This finding, of course, is in keeping with our working hypothesis that plasma-cell formation is the morphological expression of antibody and gamma-globulin

* Polio virus, mumps virus, TAB vaccine, and DPT vaccine.

synthesis and is not secondary to the local presence of large amounts of gamma globulin or antibody.

Development of bacterial-type hypersensitivity in patients with agammaglobulinemia. As mentioned above, the observation that one of our agammaglobulinemic children developed a questionably positive tuberculin reaction provoked detailed consideration of the relationship of delayed (bacterial type) hypersensitivity to the formation of circulating antibody in man. The initial study indicating that ubiquitous hypersensitivity to *Streptococcus* was uniformly absent in agammaglobulinemic patients⁵ led to the conclusion that these patients are deficient in their ability to develop delayed hypersensitivity, just as they are in their capacity to produce antibody. The observations of Zinneman *et al.*²⁹ and Seltzer³⁰ describing agammaglobulinemic patients with positive tuberculin-type reactions, coupled with the report of Porter²³ that his 1 case of agammaglobulinemia developed a positive tuberculin reaction after infection with BCG, stimulated us to investigate this relationship further. Skin sensitivity to 2,4-dinitrofluorobenzene (DNFB) is readily produced by topical application of this compound to the skin of normal human subjects in vesicant doses.^{63, 64} In these experiments 6 normal persons and 3 agammaglobulinemic patients were tested for sensitivity to DNFB. In each instance the reactions were negative. Then DNFB was applied to the back of 3 of the normal persons and 3 agammaglobulinemic patients in vesicant doses*. As a result of the latter treatment, a large blister was produced in each instance. Two weeks later the test patch† was applied to the skin of the forearm. In each "sensitized" patient, a delayed inflammatory response characteristic of bacterial-type hypersensitivity developed. This reaction was first noticeable approximately 12 hours after application of the test patch and reached a maximum 24 to 36 hours later. Simultaneously, skin tests applied to the 3 normal control subjects who were originally tested with the actively sensitized group remained negative and each of 25 normal children similarly tested showed negative reactions.

Transfer of delayed (bacterial-type) hypersensitivity by injection of leukocytes from sensitized agammaglobulinemic and normal persons. To test further the nature of the skin sensitivity to 2,4-DNFB produced in the agammaglobulinemic and immunologically normal persons, 3 additional experiments were performed. Washed leukocytes from 250 cc. of blood were obtained from a sensitized agammaglobulinemic child and a sensitized normal adult. In the first experiment, 8.4×10^6 viable leukocytes from a sensitized agammaglobulinemic patient were injected subcutaneously into a nonsensitive normal adult recipient; 48 hours later the recipient was tested for sensitivity to DNFB. Twenty-four hours after application of the skin test, an erythematous indurated reaction was present at the site of the patch. This local reaction reached a maximum 48 hours later and finally subsided approximately 5 days after application of the skin-test patch. Seventy-two hours after application of the patch, an angry erythematous flare reaction occurred in the skin and subcuta-

* Application was made with an 0.5-cm. filter pad wet with a 10 per cent solution of DNFB in a vehicle of 1 part olive oil and 9 parts acetone.

† Application was made with an 0.5-cm. filter pad wet with 0.002 M solution of DNFB in the same vehicle.

neous tissue at the site of injection of the leukocytes from the sensitized agammaglobulinemic donor. An entirely similar result was obtained when 5.0×10^8 washed viable leukocytes from a sensitized normal person were injected subcutaneously into another nonsensitized recipient. The final normal adult volunteer was skin tested 3 times in parallel with the normal volunteers who were sensitized both actively and passively. In no instance did a significant reaction occur on the skin of this person.

Antibody production following transplantation of lymph nodes to patients with agammaglobulinemia. Following successful transplantation of skin to patients with congenital agammaglobulinemia, it was considered worth while to attempt to homotransplant lymphoid tissue from normal persons to the agammaglobulinemic patient in an effort to restore their immunological reactivity. Thus far we have performed 2 such experiments. In the first, a single inguinal lymph node was transplanted into the abdominal wall of a young child with congenital agammaglobulinemia. In another, 5 large lymph nodes were transplanted into the abdominal wall of an adult with acquired agammaglobulinemia. In the first experiment, no demonstrable gamma globulin or antibody was formed, and subsequent antigenic stimulation did not give rise to antibody production. Since this was a negative experiment, it will not be considered further. In the second experiment, no demonstrable change in gamma-globulin concentration occurred following implantation of the nodes, but agglutinins were produced in response to antigenic stimulation, and circulating antibody was detectable for 2 months following the transplantation. Subsequent to return of antibody titer to zero, antigenic stimulation was again provided. On this occasion, no agglutinins were formed in response to injections of antigen. In spite of the clearly demonstrable immune response following homotransplantation of lymph nodes to this patient, no significant change in the level of gamma globulin was observed. Evaluation of the gamma-globulin level was carried out, using an immunochemical method as well as zone and free electrophoresis.

The results of this study are summarized in FIGURE 13, where it may be seen that no agglutinins were formed in response to numerous injections of typhoid-paratyphoid vaccine prior to transplantation*. An 8-year-old child was immunized to typhoid-paratyphoid antigens by the subcutaneous injection of 1.5 cc. of vaccine in three doses over a 3-week period. In response to this stimulation, this patient showed vigorous production of agglutinins against the typhoid H, O, and *paratyphi B* antigens for which he was tested. Six weeks later, 4 days following a recall injection of 0.5 cc. of TAB vaccine into the dermal and subcutaneous tissue of the anterior medial aspect of the thigh, 5 large lymph nodes were removed from the inguinal region of this patient. One of the nodes was transplanted intact (within its capsule) to the agammaglobulinemic patient. One of the nodes was sectioned with a new razor blade into 12 small triangular pieces, each with a capsular outer edge. Two nodes were sliced into thin slices approximately 1 mm. in thickness, and 1 node was teased in normal saline so that most of the cells were released into the menstruum. After extensive teasing, the released cells were drawn into a sterile

* One half cc. TAB vaccine (Wyeth) containing typhoid bacillus, 1000 million; paratyphoid bacillus A, 250 million; and paratyphoid bacillus B, 250 million killed organisms per cc.

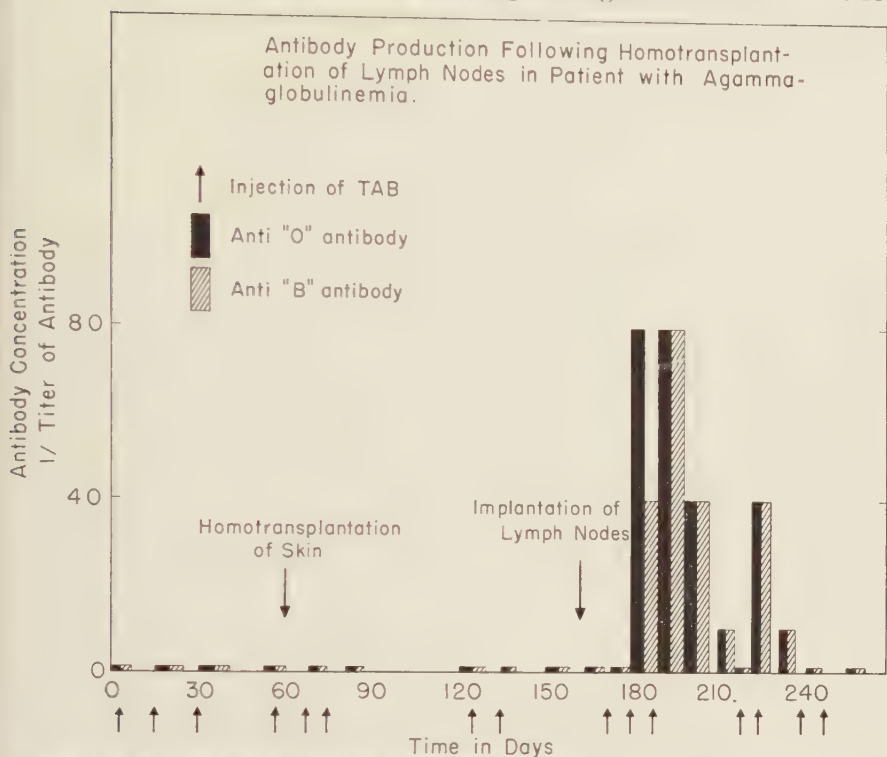


FIGURE 13. Antibody production following the homotransplantation of lymph nodes in a patient with agammaglobulinemia. Note the failure of formation of antibody following the homotransplantation of skin, and the clear-cut immune response following the transplantation of 5 lymph nodes. After forming antibody for 2 months, an immunologically unresponsive state recurred.

syringe and injected into the subcutaneous tissue of the anterior abdominal wall. In all, approximately 2.5 gm. of lymph-node tissue were transplanted into the subcutaneous tissue of the abdominal wall of the recipient agammaglobulinemic patient. As shown in FIGURE 13, no demonstrable antibody was produced during the 3 days following the transplantation of the lymph-node tissue. Following injection of TAB vaccine, however, the agammaglobulinemic patient who had been transplanted with lymph nodes from a normal person produced agglutinins against the H and O antigens of the typhoid bacillus and the B antigen of the paratyphoid organism in a maximum titer of 1:80. After return of antibody titers to zero levels approximately two months following initial implantation of the nodes, the immune response could no longer be elicited. We interpret these observations to indicate that transplantation of normal lymph nodes into an adult with acquired agammaglobulinemia temporarily restores a definite but probably minimum immunological responsiveness. Unfortunately, and possibly as a result of the studies performed in this effort to reconstitute his immunological capacity, the 58-year-old man to whom the lymph nodes were transplanted developed fulminating hepatitis 3 months

subsequent to the lymph-node transplant and died in hepatic coma. Massive gamma-globulin therapy (150 cc.) was of no avail.

It is possible to draw a number of conclusions from these experiments. In the first place, it appears from these data that transplantation of lymphatic tissue may render an agammaglobulinemic patient immunologically responsive. Second, either for technical reasons or, perhaps more likely, because the mechanism of transplantation rejection has been invoked by either the node itself or the patient, lymph-node transplantation in the manner of this experiment does not provide sufficiently intense or sufficiently prolonged immunological reactivity to promise practical therapy for patients with acquired agammaglobulinemia. Studies by Martin,⁶⁵ however, indicate that immunological capacity lasting even longer (160 days) may be produced by lymph-node transplant in these patients. In the light of the studies on skin transplantation, it seems possible that a more rewarding response may occur in children with congenital agammaglobulinemia. The persistence of disease, even with replacement gamma globulin and prophylactic antibiotic therapy, provokes continued consideration of transplantation as a means of correcting the metabolic defect of these patients. Bone-marrow infusion, cancellous bone transplantation, and production of splenosis peritonei seem to offer possible approaches to this end.

From our experience, however, it seems necessary to derive a warning. Although the hepatitis occurring in this patient might have been brought about as a result of contact with subclinical or overt hepatitis or could have been introduced by any of several hundred injections utilized in his treatment and investigative workup, its possible relationship to the transplantation should provoke serious thought. As with blood transfusions, it may be possible to introduce homologous-serum hepatitis by transplantation of tissues or organs, and this possibility must be taken into account whenever transplantation is considered in man.

The occurrence of pregnancy in a patient with acquired agammaglobulinemia. The final transplantation that has been studied in the agammaglobulinemic patients is a transplantation of nature. Pregnancy occurred in a 30-year-old agammaglobulinemic female with acquired agammaglobulinemia. Prior to her pregnancy it had been established that this patient's disease was associated with a marked gamma-globulin deficit, and that she failed to form antibody in response to the most intensive stimulation with a wide variety of antigens. Indeed, her gamma-globulin level (determined immunochemically) ranging from 10 to 13 mg. per cent was as low as that of most of the children with sex-linked congenital agammaglobulinemia whom we have studied. In addition, so far as could be determined, her "immunological paralysis" was complete. During the first trimester of her pregnancy, before it was realized that she was pregnant, the patient received antigenic stimulation with a wide variety of antigens, including multiple injections of TAB vaccine, diphtheria, pertussis, and tetanus antigens, mumps vaccine, polio vaccine, WEE vaccine, several rickettsial vaccines, and mismatched blood. In no instance was detectable antibody formed against these stimuli. When it was discovered that the patient was pregnant, no further attempts at immunization were made, with two exceptions. In

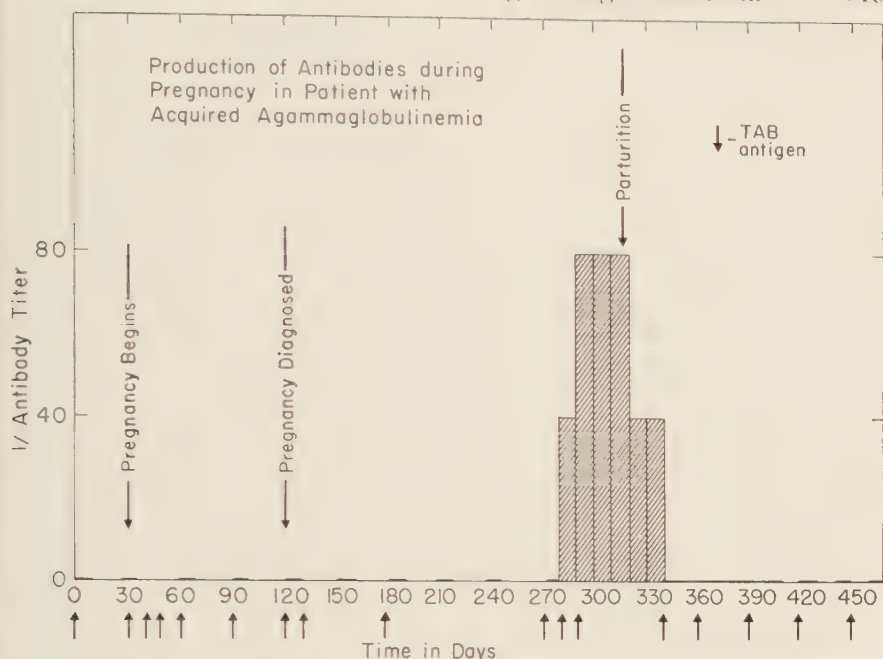


FIGURE 14. Summarized here are immunological studies carried out on an agammaglobulinemic patient during pregnancy. Note the failure of antibody response prior to pregnancy, during the first and second trimester, and following delivery. Formation of typhoid-paratyphoid antibodies in response to immunization during the last month of pregnancy, however, is indicated. Stimulation with other antigens in the immediate *post partum* period indicated the loss of immunological responsiveness immediately after parturition.

the second trimester (midway in the fifth month) 0.5 cc. of TAB vaccine was administered, and blood samples were taken weekly for 3 weeks. During the final month of pregnancy another attempt was made to immunize the patient. Three injections of TAB vaccine were given at weekly intervals. Blood samples were drawn prior to and at weekly intervals after starting the series of injections. Two days prior to delivery, the patient was injected subcutaneously with 1 cc. of mumps vaccine (Lederle). Blood samples were taken at weekly intervals throughout the first and third trimesters and as indicated during the 5th and 10th months. Bone-marrow studies were performed prior to pregnancy, during attempted immunization prior to pregnancy, repeatedly during the first trimester of pregnancy, before and after attempted immunization during the second trimester, and prior to and during immunization during the third trimester. In addition, biopsies of the patient's lymph nodes draining the site of an antigenic stimulation were taken prior to pregnancy, during the first trimester, during the third trimester, at the time of delivery, and 3 months following delivery. Imprints of the nodes were made and stained with Wright-Giemsa, and sections prepared in the usual way were stained with methyl-green pyronine and hematoxylin eosin. The results of this study are summarized in FIGURE 14. It will be seen that no antibody was produced to antigenic stimulation with TAB vaccine prior to pregnancy or during the first or second trimester. During the final month, however, agglutinins were formed

against all 3 of the antigens tested (H, O, and B) in titers as high as 1:80. Following parturition, in spite of weekly injections of 0.5 cc. TAB vaccine, the antibody titer rapidly declined. Indeed, no detectable agglutinins could be found 3 weeks following delivery. Stimulation with a multiplicity of other antigens following parturition revealed the immediate return to a state of immunological unresponsiveness following delivery of the baby and placenta. The stimulus provided by the mumps antigen just prior to parturition did not give rise to the formation of complement-fixing antibodies at any time. No significant increment in gamma-globulin concentration, as revealed by paper or free electrophoresis, or by immunochemical methods, occurred at any time during pregnancy or after delivery.

Studies of the bone marrow and nodes draining the site of antigenic injection did not reveal any detectable differences prior to pregnancy, during the first, second, or third trimesters, at parturition, or 3 months *post partum*. These tissues were studied particularly for evidence of plasma-cell development that might be interpreted as the morphological sign of antibody production. In none of the biopsies could plasma cells be found.

These observations, together with the facts that antibody production occurred only during the third trimester and that the immunological reactivity was rapidly lost following parturition, led us to consider it likely that the product of the pregnancy—either the child or the placenta—was responsible for the observed antibody production.

Study of the placenta was of interest. Crude saline extracts of the placenta revealed agglutinins against the H and O antigens in a 1:40 titer. In addition, microscopic study of the placenta revealed basophilic reticulum (mesenchymal) cells, particularly in the villi. Some of these cells possessed morphological features making them indistinguishable from plasma cells. The latter were pyraninophilic. From these observations it seems at least possible that placental participation was responsible for the antibody production observed during the final month of pregnancy.

Study of gamma-globulin and antibody formation during the first year of life by an infant born of an agammaglobulinemic mother. Complementary to the observations made on the pregnant agammaglobulinemic female are the studies done on her child during the neonatal period and throughout the first year of life. Blood samples were taken from the cord blood and by *vena* puncture from the baby at 2, 4, 6, 7, 10, 17, 24, 30, 42, 50, 60, and 90 days of life and monthly thereafter throughout the first year. Gamma-globulin concentration was determined by paper and free-electrophoresis methods, as well as by an immunochemical technique; total proteins were determined using a standard biuret method. Antigenic stimulation was provided weekly by injection of TAB vaccine and DPT vaccine during the first 2 months and monthly thereafter by injection of TAB and DPT antigens. Antibody determinations, including the following, were carried out on the blood samples that were available: H, O, and B agglutinins, complement fixation antibodies against mumps, diphtheria antitoxin, Schick test, and isoagglutinins against heterologous blood groups. The observations, some of which are recorded on FIGURE 15, may be summarized as follows:

Gamma globulin and antibody production in baby born of an agammaglobulinemic mother.

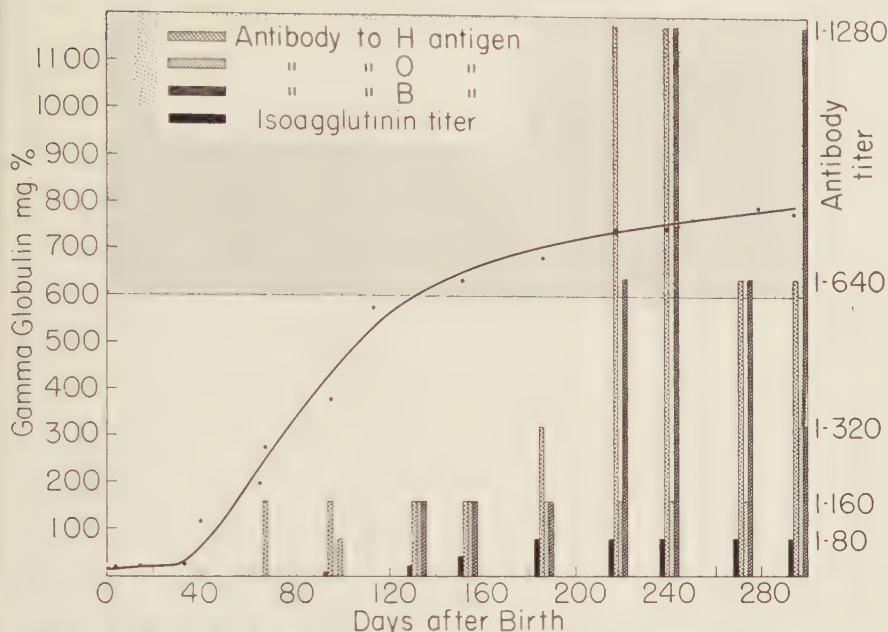


FIGURE 15. Gamma globulin and antibody production in a baby born of an agammaglobulinemic mother. Note that, in spite of intensive antigenic stimulation, the baby failed to form antibody and gamma globulin in the first few weeks of life. Following the beginning formation of gamma globulin, the baby formed antibodies both naturally and in response to antigenic stimulation.

The baby was born essentially agammaglobulinemic (11 mg. gamma globulin by the immunochemical method) and remained agammaglobulinemic during the first 6 weeks of life. In contradistinction to the maternal serum, the serum from the cord blood lacked demonstrable agglutinins against typhoid and paratyphoid antigens. (This result was to be suspected if these antibodies were derived from a maternal source, since these agglutinins do not traverse the placental barrier.) This baby showed no demonstrable immune response until 2 months of age, 18 days following the first increment of gamma globulin as revealed by the immunochemical technique. Thereafter, the baby formed agglutinins against the H, O, and B antigens, formed diphtheria antitoxin, and became Schick-negative at 4 months of age. As in normal infants, this baby, who was of blood group O, began to show significant isoagglutinin titers against A cells 3 months after birth, and thereafter a steady increase in anti-A agglutinins occurred. At 1 year of age the baby possessed the respectable titer against A cells of 1:1280 in saline. The paper-electrophoretic patterns revealed the appearance of gamma globulin at approximately 60 days of age, 18 days after significant amounts were first demonstrated by the more sensitive immunochemical method. The concentration of gamma globulin in the serum steadily increased in amount, so that by the time the baby was 10 months of age the electrophoretic pattern appeared to be normal.

Of real interest have been the changes occurring in the bone marrow and lymphatic tissue during the first year of life. Bone-marrow and lymph-node biopsies were taken immediately after parturition; at 6 days of age (4 days following subcutaneous and intradermal injections of DPT and TAB antigens); at 3 months following comparable antigenic stimulation; and again at 14 months of age, 4 days following injection of the same antigens.

The lymph nodes taken from the inguinal region at birth were small and poorly developed, had poorly formed primary and secondary follicles and, indeed, resembled the lymph nodes of patients with congenital agammaglobulinemia. At 6 days of age, following intensive antigenic stimulation, the nodes were somewhat larger, showed feeble attempts at primary follicle formation and some degree of proliferation of the stromal reticulum. Extensive study of both bone marrow and lymph nodes with high-power microscopy at birth and at 6 days of age revealed complete failure of plasma-cell formation. The lymph node removed following antigenic stimulation at 3 months of age, when a definite immune response was obtained, was distinctly different from the nodes described above. Primary follicles were well-formed, and a few well-differentiated secondary follicles were present in the cortex. In addition, plasma-cell formation in the medullary portion of the node was demonstrated, as was plasma-cell formation in the mantle zone about the secondary follicles. The node taken following antigenic stimulation at 3 months of age was, however, still relatively small, had relatively few secondary follicles, and showed a plasma-cell response similar to that observed during a primary response rather than the profuse plasma-cell response usually associated with a secondary or tertiary stimulation, which had been provided. At this time the bone marrow, although containing plasma cells in contradistinction to the marrows obtained in the neonatal period, which contained none, still possessed relatively few of these elements when compared to the marrow of an older child undergoing comparable antigenic stimulation.

At 14 months, evidence of further maturation of the lymphoid tissue was observed. The node removed 4 days following antigenic stimulation at this time was large in size (approximately 0.5 gm.) and possessed all of the characteristics of a node from a mature person responding to secondary or tertiary antigenic stimulation. The cortex was thick, with well-developed primary and secondary follicles. The medullary cords were relatively thick, and high-power microscopy revealed the presence of large clusters of plasma cells and developing plasma cells among the elements in the medullary cords, as well as unorganized areas in the cortex and in the mantle zone of the secondary follicles. Furthermore, plasma cells could now be found with relative ease among the cellular elements of the bone marrow.

In this infant we have had the opportunity of studying one phase of the development of immunological responsiveness in an immunologically normal child born of an agammaglobulinemic mother. The child's failure to form gamma globulin and the immunological unresponsiveness observed during the first few weeks of life are not at variance with the observations of others^{18, 66-73} studying immune response and gamma-globulin metabolism during the first year of life in normal infants. The advantage of our clinical opportunity

lay in the fact that this child was born of a mother who was producing only minimal amounts of gamma globulin. The observations lend support to the conclusion that the high gamma-globulin level usually observed in normal newborn infants^{72, 73, 74} is probably derived entirely from the mother, with a possible small contribution from placental synthesis.⁷¹ Further, it would appear that, during the neonatal life in man, there occurs a period of immunological unresponsiveness (probably variable in duration) that is correlated with an unresponsiveness of the reticulum (mesenchyme) to antigenic stimulation.

The establishment of this period of immunological unresponsiveness in the human infant is immediately provocative. It invites one to attempt to reproduce, in humans, Woodruff's rat experiment,⁷⁵ in which he produced acquired tolerance⁷⁶ to skin homotransplantation by the injection of leukocytes on the first day of life. Investigations in this direction are contemplated.

Comment

Proper discussion of all the points presented in this paper would unconscionably lengthen it. Consequently, discussion of points of controversy and the proper relation of each of these observations to the findings of others will be left for the papers in which the details of each of the observations are reported. Several points having particular reference to transplantation, however, should be emphasized. It is clear from our observations, particularly on the patients with acquired agammaglobulinemia, that low levels of gamma globulin alone will not permit homotransplantation. The data suggest, on the other hand, that the profound disturbance of immunological mechanism associated with the congenital, sex-linked form of agammaglobulinemia is sufficient to permit the prolonged survival of homotransplanted skin in these patients. As will be seen from a review of other papers in this volume, from the observations of Porter, and from our own studies of bacterial-type hypersensitivity in patients with agammaglobulinemia, the successful transplantation in these patients appears to introduce a conflict. Much evidence, the bulk of which has accumulated only recently, suggests that the immunological basis of homotransplantation failure more closely resembles delayed (bacterial-type) hypersensitivity than immunity associated with production of circulating antibody. Yet the same agammaglobulinemic patient who retains homotransplanted skin appears to be able to develop delayed sensitivity to tuberculin and certain chemicals (DFNB, for example) when properly stimulated. The explanation for this paradox is not immediately apparent, but a number of experimental approaches are available for its resolution. Perhaps both a circulating and cellular factor are necessary for transplantation failure, and perhaps the delayed sensitivity responsible for transplantation failure resembles the tuberculin-type reaction in that it involves cells, but is not identical to it. It is possible, then, that the agammaglobulinemic child might fail in the development of such transplantation sensitivity, as he does in the response to antigens and in the immunological adjustment to the histocompatibility factors that mark the red blood cells. Perhaps our original observations on sensitivity to strep-

tococcal and pneumococcal products signify, as we originally thought, that there exists a quantitative deficiency in the capacity to develop bacterial-type hypersensitivity in these patients. It may be that this hypothesis can be tested with Uhr, Salvin, and Pappenheimer's new methods for study of bacterial-type hypersensitivity. It is even possible that an as-yet-undefined circulating antibody, perhaps one that is quickly removed from the circulation by the transplant, plays a more important role in transplantation failure in man than is presently considered likely. Whatever the explanation of this apparent paradox may be, its solution could contribute greatly to our understanding of transplantation failure. It also is clear that more must be learned concerning the nature of bacterial-type hypersensitivity and its relationship to other immune phenomena in man and animals. Indeed, it is our conclusion that here lies the core of the transplantation problem.

It is certain that our observations do not exhaust the potential of transplantation in the management of agammaglobulinemia. The fact that a significant immune response was produced in one of these patients, in many ways among the least favorable of the subjects in this group, stimulates one to proceed with further effort in this direction. Martin⁶² obtained immunological responsiveness over a much longer period in the agammaglobulinemic adult to whom he transplanted a greater quantity of lymphatic tissue. We should not be discouraged by the fact that ultimate immunological failure recurred, since his patient, having acquired agammaglobulinemia, might be expected to be as unsatisfactory for permanent homotransplantation as was our adult case. Even realizing the danger of hepatitis mentioned earlier, the persistent disability, together with the potential hazard of their disease (in spite of gamma-globulin injections and antibiotic therapy), make urgent a continued effort to provide these patients with immunological reactivity. Certainly, from this viewpoint, transplantation deserves further study.

Summary and Conclusions

(1) Congenital agammaglobulinemia is a disease featured by failure of gamma-globulin production and failure of immunological response to antigenic stimulation. The disorder appears to be an inborn error of metabolism generally transmitted as a sex-linked recessive trait occurring only in males.

(2) Acquired agammaglobulinemia may begin in either sex at any age and tends to be less complete than the congenital form of disease. No evidence of any hereditary pattern has as yet been discovered.

(3) Both congenital and acquired isolated agammaglobulinemia are associated with a disturbance in function of the mesenchyme that may be reflected as diverse hematological abnormalities. This mesenchymal deficiency is regularly reflected in failure of the development of plasma cells and pyroninophilic cells in response to primary, secondary, and tertiary antigenic stimulation.

(4) Normal children and adults develop a plasma-cell and pyroninophilic cellular response to antigenic stimulation in the lymph node draining the site of an intradermal injection of antigen and in the bone marrow following intradermal or intravenous injection of antigen.

(5) No circulating factor capable of inhibiting the immune response has been found in patients with agammaglobulinemia.

(6) Eight patients with agammaglobulinemia were found to lack bacterial-type hypersensitivity to streptococcal antigens, whereas the majority of normal children and adults of comparable age group possessed this sensitivity.

(7) The intravenous and subcutaneous injection of 1 to 2 billion viable white blood cells from the peripheral blood of persons previously immunized to typhoid-paratyphoid agglutinins and diphtheria toxoid failed to introduce capacity to develop active immunity in 3 patients with agammaglobulinemia.

(8) Transplantation of skin in both full and split thickness to two patients with congenital agammaglobulinemia resulted in prolonged and possibly permanent survival of the grafts. In one instance, the successful "take" has remained for 23 months. In the other it has remained 14 months.

(9) Transplantation of skin in full thickness to a patient with acquired agammaglobulinemia and "immunological paresis" resulted in a successful initial "take" and a gradual slough that occurred between 10 and 16 weeks after application.

(10) Transplantation of skin from patients with agammaglobulinemia to immunologically normal persons has been unsuccessful in 4 instances.

(11) Transplantation of skin from a normal person to a patient with transient agammaglobulinemia of infancy resulted in slough 24 days after application.

(12) Transplantation of a single lymph node from a subject actively forming antibodies failed to provide a patient suffering from congenital agammaglobulinemia with antibodies or with immunological capacity.

(13) Transplantation of 4 lymph nodes from a previously sensitized patient provided immunological reactivity to a patient with acquired agammaglobulinemia that lasted $2\frac{1}{2}$ months, but then ceased.

(14) Pregnancy occurred in an adult female with acquired agammaglobulinemia. This patient failed to produce antibody in response to antigenic stimulation prior to her pregnancy and during the first and second trimesters. During the third trimester, however, agglutinins were formed after stimulation with typhoid-paratyphoid vaccine.

(15) The baby born of this pregnancy was essentially agammaglobulinemic during the first 50 days of the neonatal period, following which production of gamma globulin accompanied by development of immunological capacity was demonstrated.

(16) Although serial bone-marrow and lymph-node biopsies of the mother were studied throughout the pregnancy, no plasma cell development was observed.

(17) Plasma cells were absent from the baby's bone marrow and lymph nodes during the neonatal period and did not develop in either location in response to antigenic stimulation.

(18) When the baby began to form antibody during the third postpartum month, antigenic stimulation resulted in a responsive formation of plasma cells and secondary follicles in the lymph nodes, and of plasma cells in the bone marrow.

References

1. BRUTON, O. C. 1952. Agammaglobulinemia. *Pediatrics*. **9**: 722.
2. BRUTON, O. C., L. APT., D. GITLIN & C. A. JANEWAY. 1952. Absence of serum gamma globulins. *Am. J. Diseases Children*. **84**: 632.
3. JANEWAY, C. A., L. APT. & D. GITLIN. 1953. Agammaglobulinemia. *Trans. Assoc. Am. Physicians*. **66**: 200.
4. JEAN, R. 1953. Hypo- ou agammaglobulinémie isolée chez l'enfant. *Presse méd.* **61**: 40.
5. GOOD, R. A. 1954. Agammaglobulinemia: a provocative experiment of nature. *Bull. Univ. Minn. Hospital & Minn. Med. Foundation*. **26**: 1.
6. MONCKE, C. 1954. Essentielle agammaglobulinemia. *Schweiz. med. Wochschr.* **84**: 1033.
7. PRASAD, A. S. & D. W. KOSA. 1954. Agammaglobulinemia. *Ann. Internal Med.* **41**: 629.
8. BARNETT, H. L., C. W. FORMAN & H. D. LAUSON. 1952. The nephrotic syndrome in children. *Advances in Pediat.* **5**: 53.
9. YOUNG, I. I., W. Q. WOLFSON & C. COHN. 1955. Studies in serum proteins: agammaglobulinemia in the adult. *Am. J. Med.* **19**: 222.
10. KREBS, E. G. 1946. Depression of gamma globulin in hypoproteinemia due to malnutrition. *J. Lab. Clin. Med.* **31**: 85.
11. THOMPSON, W. H., M. R. ZIEGLER & I. McQUARRIE. 1932. Comparative study of the inorganic metabolism in nephrosis and in edema of undetermined origin. *Am. J. Diseases Children*. **44**: 650.
12. SCHICK, B. & J. W. GREENBAUM. 1945. Edema with hypoproteinemia due to congenital defect in protein formation. *J. Pediat.* **27**: 241.
13. FRIED, C. T. & W. L. HENLEY. 1954. Deficiency of gamma globulin with edema and hypoproteinemia. *Pediatrics*. **14**: 59.
14. ULSTROM, R. A., N. J. SMITH & L. ZELDIS. 1954. Multiple plasma protein defects in an infant. *Proc. Western Soc. Pediat. Research.* : 12.
15. GOOD, R. A., W. F. MAZZITELLO & S. J. ZAK. Studies on agammaglobulinemia. VIII. Immunologic, biochemical and hematologic investigations of agammaglobulinemia during pregnancy. To be published.
16. GOOD, R. A. & S. J. ZAK. Disturbances of gamma globulin synthesis as experiments of nature. *Pediatrics*. In press.
17. MOORE, D. H., R. MARTIN DU PAN & C. L. BUXTON. 1949. An electrophoretic study of maternal and infant sera. *Am. J. Obstet. Gynecol.* **57**: 312.
18. ORLANDINI, T. O., A. SASS-KORTSAK & J. H. EBBS. 1955. Serum gamma globulin levels in normal infants. *Pediatrics*. **16**: 575.
19. GOOD, R. A. & V. C. KELLEY. Serum gamma globulin levels from birth to maturity. Unpublished data.
20. SPAIN, D. M., V. A. BRADESS & I. J. GREENBLATT. 1954. Possible factor in sudden and unexpected death during infancy. *J. Am. Med. Assoc.* **156**: 246.
21. GOOD, R. A. & R. L. VARCO. 1955. A clinical and experimental study of agammaglobulinemia. *J. Lancet*. **75**: 245.
22. GOOD, R. A. 1954. Agammaglobulinemia— an experimental study. *Am. J. Diseases Children*. **88**: 625.
23. PORTER, H. 1955. Congenital agammaglobulinemia— a sex linked genetic trait and demonstration of delayed skin sensitivity. *Am. J. Diseases Children*. **90**: 617.
24. GITLIN, D. 1955. Low resistance to infections, relationship to abnormalities in gamma globulin. *Bull. N. Y. Acad. Med.* **31**: 359.
25. GOOD, R. A. & S. J. ZAK. Immunochemical study of the gamma globulin concentration in patients with agammaglobulinemia. Unpublished manuscript.
26. LANG, N., G. SCHETTLER & R. WILDHACK. 1954. Über einen Fall von Agammaglobulinämie und das Verhalten parenteral zugeführten radioaktiv markierten Gammaglobulins im Serum. *Klin. Wochschr.* **32**: 856.
27. ARMSTRONG, S. H., J. KUKRAL, J. HERSHMAN, K. McLEOD, J. WOLTER & D. BRONSKY. 1954. Comparison of the persistence in the blood of gamma globulins labeled with S^{35} and I^{131} in the same subjects. *J. Lab. Clin. Med.* **44**: 762.
28. GOOD, R. A. & W. F. MAZZITELLO. 1956. Chest diseases in patients with agammaglobulinemia. *Diseases of the Chest*. **24**: 9.
29. ZINNEMAN, H. H., W. H. HALL & B. I. HELLER. 1954. Acquired agammaglobulinemia. *J. Am. Med. Assoc.* **156**: 1390.
30. SELTZER, S., S. BARRON & M. TAPOREK. 1955. Idiopathic hypogammaglobulinemia and agammaglobulinemia. *New Engl. J. Med.* **252**: 255.

31. KULNEFF, V. N., K. O. PEDERSEN & J. WALDENSTRÖM. 1955. Drei Fälle von Agammaglobulinämie; ein klinischer, genetischer, und physikalisch-chemischer Beitrag zur Kenntnis des Proteinstoffwechsels. *Schweiz. med. Wochschr.* **85**: 363.
32. KEIDEN, S. E., K. MCCARTHY & J. C. HAWORTH. 1953. Fatal generalized vaccinia with failure of antibody production and absence of serum gamma globulin. *Arch. Disease Childhood.* **28**: 110.
33. YOUNG, I. I. & W. Q. WOLFSON. 1954. The agammaglobulinemic syndrome of young men. Its differentiation into familial lymphopenic agammaglobulinemia and familial non-lymphopenic dysgammaglobulinemia. *Clin. Research. Proc.* **2**: 101.
34. RHON, R. J., R. H. BEHNKE & W. H. BOND. 1954. Acquired agammaglobulinemia with hypersplenism. *J. Lab. Clin. Med.* **44**: 918.
35. KOLOUCH, R., JR. 1938. Origin of bone marrow plasma cells associated with allergic and immune states of rabbits. *Proc. Soc. Exptl. Biol. Med.* **39**: 147.
36. KOLOUCH, R., JR., R. A. GOOD & B. CAMPBELL. 1947. The reticuloendothelial origin of the bone marrow plasma cell in hypersensitive states. *J. Lab. Clin. Med.* **32**: 749.
37. GOOD, R. A. 1950. Experimental allergic brain inflammation: a morphologic study. *J. Neuropathol. Exptl. Neurol.* **9**: 78.
38. GOOD, R. A. & B. CAMPBELL. 1950. Relationship of bone marrow plasmacytosis to the changes in serum gamma globulin in rheumatic fever. *Am. J. Med.* **9**: 330.
39. GOOD, R. A. 1948. Effect of passive sensitization and anaphylactic shock on rabbit bone marrow. *Proc. Soc. Exptl. Biol. Med.* **67**: 203.
40. CAMPBELL, B. & R. A. GOOD. 1950. Cytopathology of the brain and reticuloendothelial organs in allergic encephalitis in guinea pigs. *Arch. Neurol. Psychiat.* **63**: 298.
41. CAMPBELL, B. & R. A. GOOD. 1949. Antigen-antibody mechanisms in neurotropic virus disease. *Ann. Allergy* **7**: 471.
42. GOOD, R. A. 1954. Absence of plasma cells from bone marrow and lymph nodes following antigenic stimulation in patients with agammaglobulinemia. *Rev. Hémat.* **9**: 502.
43. GOOD, R. A. 1955. Studies on agammaglobulinemia. II. Failure of plasma cell formation in the bone marrow and lymph nodes of patients with agammaglobulinemia. *J. Lab. Clin. Med.* **46**: 167.
44. BJOERNEBOE, M. & H. GORMSEN. 1943. Experimental studies on the role of plasma cells as antibody producers. *Acta Pathol. Microbiol. Scand.* **20**: 649.
45. FAGRAEUS, A. 1948. Antibody production in relation to the development of plasma cells. *Acta Med. Scand. Suppl.* **130**: 7-122.
46. EHRRICH, W. E., D. L. DRABKIN & C. FORMAN. 1949. Nucleic acids and the production of antibody by plasma cells. *J. Exptl. Med.* **90**: 157.
47. McMASTER, P. D. 1953. The sites of antibody production. *In* *Nature and Significance of the Antibody Response*. A. M. Pappenheimer, Ed. : 14. Columbia Univ. Press. New York, N. Y.
48. COONS, A. H., E. H. LEDUC & J. M. CONNOLLY. 1955. Studies on antibody production. I. A method for histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exptl. Med.* **102**: 49.
49. LEDUC, E. H., A. H. COONS & J. M. CONNOLLY. 1955. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exptl. Med.* **102**: 61.
50. BARR, D. 1951. The function of the plasma cell. *Am. J. Med.* **9**: 277.
51. RUNDLES, R. W., E. V. COONRAD & T. ARENDS. 1954. Serum proteins in leukemia. *Am. J. Med.* **16**: 842.
52. LOEB, V. Personal communication.
53. GOOD, R. A. & V. C. KELLEY. 1955. Studies on agammaglobulinemia. V. The role of the adrenal gland in the phenomenon. *Proc. Soc. Exptl. Biol. Med.* **88**: 99.
54. FRICK, P. G. & R. A. GOOD. Studies on agammaglobulinemia. VI. Hemostasis in patients with agammaglobulinemia. *Proc. Soc. Exptl. Biol. Med.* In press.
55. WEDGEWOOD, R. J. P. & C. H. JANEWAY. 1953. Serum complement in children with collagen diseases. *Pediatrics.* **11**: 569.
56. MCQUARRIE, I. 1944. *Experiments of Nature and Other Essays*. Univ. Kansas Press. Lawrence, Kans.
57. GOOD, R. A. & R. L. VARCO. 1955. Studies on agammaglobulinemia. I. Successful homograft of skin in a child with agammaglobulinemia. *J. Am. Med. Assoc.* **167**: 713.
58. VARCO, R. L., L. D. MACLEAN, J. B. AUST & R. A. GOOD. 1955. Agammaglobulinemia: an approach to homovital transplantation. *Ann. Surg.* **142**: 355.
59. BOYD, W. C. 1947. *Fundamentals of Immunology*. Interscience Publ., Inc. New York, N. Y.

60. GRABAR, P. & C. A. WILLIAMS. 1953. Méthode permettant l'étude conjuguée des propriétés électrophorétiques et immuno-chimiques d'un mélange de protéines. Application au sérum sanguin. *Biochim. et Biophys. Acta*, **10**: 196.
61. SLATER, R. J., S. M. WARD & H. G. KUNKEL. 1955. Immunological relationships among the myeloma proteins. *J. Exptl. Med.* **101**: 85.
62. PAULING, L. 1940. Theory of the structure and process of formation of antibodies. *J. Am. Chem. Soc.* **62**: 2643.
63. STETSON, C. A. Personal communication.
64. GOOD, R. A. & S. J. ZAK. Unpublished data.
65. MARTIN, C. H. 1957. Second Tissue Homotransplantation Conference. *N. Y. Acad. Sci.* **64**(5): 927.
66. WIENER, A. H. BERGER & S. LENKE. 1951. Serum gamma globulin in infants. *Lab. Digest*, **14**: 11.
67. WIENER, A. S. 1951. The half-life of passively acquired antibody globulin molecules in infants. *J. Exptl. Med.* **94**: 213.
68. GOOD, R. A. Studies on the relationship of plasma cell development to formation of serum gamma globulin and antibodies in the human infant. Unpublished manuscript.
69. SLATER, R. J. 1954. Investigation of an infant born of a mother suffering from cirrhosis of the liver. *Pediatrics*, **13**: 308.
70. OSBORN, J. J., J. DANCIS & J. F. JULIA. 1952. Studies on the immunology of the newborn infant. I. Age and antibody production. *Pediatrics*, **9**: 736.
71. DANCIS, J., J. J. OSBORN & H. W. KUNS. 1953. Studies on the immunology of the newborn infant. IV. Antibody formation in the premature infant. *Pediatrics*, **12**: 151.
72. DANCIS, J., N. BRAVERMAN & B. KATCHEN. 1955. The synthesis of serum proteins by human placenta. *Am. J. Diseases Children*, **90**: 558.
73. LONGSWORTH, L. G., R. M. CURTIS & R. J. PEMBROKE, JR. 1945. The electrophoretic analysis of maternal and fetal plasmas and sera. *J. Clin. Invest.* **24**: 46.
74. KNAPP, E. L. & J. I. ROUTH. 1949. Electrophoretic studies of plasma proteins in normal children. *Pediatrics*, **4**: 508.
75. WOODRUFF, M. F. A. & L. O. SIMPSON. 1955. Induction of tolerance to skin homografts in rats by injection of cells from the prospective donor soon after birth. *Brit. J. Exptl. Pathol.* **36**: 494.
76. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature*, **172**: 603.
77. LAWRENCE, H. G. 1955. Transfer in humans of delayed skin sensitivity to streptococcal substance and to tuberculin with leukocytes. *J. Clin. Invest.* **34**: 219.

Discussion of the Paper

ELVIN A. KABAT (*Presbyterian Hospital, Columbia University, New York, N. Y.*): I should like to point out that we have indiscriminately been using a number of terms such as "immunological paralysis" and "actively acquired tolerance" to describe various phenomena. As our knowledge of these matters is just beginning to take shape, I should like to examine the experimental basis for some of these phenomena in an effort to limit the use of certain of these terms so as to avoid any resultant confusion.

"Immunological paralysis" was first introduced by Felton, who observed that mice could be actively immunized with as little as 0.1 μ g. of the purified pneumococcal-type specific polysaccharides so that they would be resistant frequently to 10^6 lethal doses of living virulent microorganisms of the type used for immunization. If, however, instead of this minute dose, Felton injected 500 μ g.—5000 times as much antigen—into each mouse, he noted that the animals never developed immunity to challenge with pneumococci. Subsequent studies by Felton, Stark, Dixon, Maurer, and Weigle, and also by Coons and his co-workers, have demonstrated that a characteristic of this phenomenon is persistence of the pneumococcal polysaccharide in the tissues for very long periods of time, essentially for the entire life of the mouse. Measurements

of the turnover of labeled homologous rabbit antibody injected into such mice have shown that, as antibody is metabolized, the polysaccharide is set free and becomes capable once again of combining with more antibody. The most likely explanation for these findings is that the polysaccharide, which is not metabolized at a significant rate, is present in sufficient quantity so that, no matter how rapidly the antibody-forming mechanism of the animal works, it is never able to create a state of antibody excess, since all antibody is continuously removed by the polysaccharide. After so many years of use, it is perhaps too late to question the appropriateness of the term "immunological paralysis" in describing this phenomenon. I believe it is here to stay. Until we have evidence that other types of observations are similar in nature and mechanism, however, we should restrict the use of this term as outlined.

The second important set of observations was made by Billingham, Brent, and Medawar, who introduced the term "actively acquired tolerance" to describe their findings. This term also has become widely adopted—indeed, far too widely—since it has been applied to observations that are, in my opinion, susceptible to other interpretations. The experimental prototype for actively acquired tolerance is the finding that embryonic animals injected with living cells of a heterologous strain grow to maturity and become able specifically to tolerate skin grafts from individuals of the strain used to provide the injected cells. The studies of Owen on nonidentical bovine twins with 2 sets of blood groups and the reports of 2 human blood-group chimeras in sets of nonidentical twins are also probable examples of this phenomenon. In these instances the mechanism appears to be quite different from that of immunological paralysis as defined above in that there is continuous proliferation of the injected cells, probably for the entire life span of the animal. Also in contrast to immunological paralysis, there is evidence that antibody formation to the cells of the heterologous donor does not occur, since, if a lymph node from another animal of the recipient strain that has rejected homografts of the donor strain is implanted into one of the tolerant animals, the graft is no longer tolerated and degenerates. This finding indicates that tolerant animals retain grafts only in the absence of antibody formation. While it is probable that the marrow transfusions in irradiated animals described earlier in these pages are of a similar nature, I am not at all convinced by any of the experimental data purporting to show that "actively acquired tolerance" has been produced to inanimate antigens. I was especially pleased to hear Melvin Cohn's presentation, from which it appears that these findings stem largely from a failure to take into account the longer persistence of large doses of antigen.

The third clearly defined phenomenon termed "agammaglobulinemia" has been very excellently described by Robert A. Good at this session. This condition, which involves failure to form antibody, results from an entirely different mechanism, namely, a defect in the capacity of the individual to synthesize gamma globulin and therefore of an inability to produce antibody. There is little doubt that it is an entity entirely distinct from either immunological paralysis or actively acquired tolerance, and it appears especially unfortunate that Good has used the term "immunological paralysis" in discussing agam-

maglobulinemia. I hope that he will withdraw this term, since it can cause no end of confusion.

Apart from these 3 clearly distinguishable experimental prototypes, a variety of other observations have been classified in one or another of these categories, I believe on insufficient evidence. This applies especially to the work with inanimate antigens, and Cohn has ably covered that phase. Unfortunately, such findings have been advanced as instances of "actively acquired tolerance." We have all been aware for many years that circulating antibody does not appear while antigen is present in the serum. Such a transitory excess of metabolizable antigens, used in very much larger amounts than pneumococcal polysaccharides, would be shorter in duration than that in the immunological paralysis described by Felton. Furthermore, it is a readily reproducible finding that a guinea pig, given a small sensitizing dose of a protein, will go into anaphylactic shock if challenged 6 to 8 days later. If, however, a very large dose of this antigen is used, so that it continues to be present in blood for longer periods, it may take 4 to 6 weeks or more before evidence of anaphylactic sensitivity can be demonstrated. To use such large doses in embryos or young animals and then to term the findings that could result from persistence of antigen as "actively acquired tolerance" is fraught with dangers and can lead to substantial confusion. Whether this state of antigen excess is even immunological paralysis in the sense used by Felton is dubious.

It is evident, in the present state of our knowledge, that it is desirable and important to restrict the use of these terms rather sharply until adequate information is obtained to permit additional experimental phenomena to be included in one or another of these categories. The 3 phenomena themselves are so complex that, unless they are sharply defined, accurate communication of ideas concerning them will become impossible.

ROBERT A. GOOD: Concerning Kabat's discussion of terminology, I should be most pleased to withdraw my use of the term "immunological paralysis" in favor of Felton's use of this epithet. I do not believe, however, that Felton's original use of the term was intended to be more than descriptive; certainly, it was not my intent to imply understanding of mechanisms involved in the immunological unresponsiveness of the agammaglobulinemic patient.

As a warning against rigidly applying a descriptive term to a biological phenomenon, one has only to recognize that the phenomenon that looked to Felton like an "immunological paralysis" now appears to Dixon and others more like a person running full speed on a treadmill and never progressing.

Since descriptive terminology can be so disconcerting, and since some of us exhibit a greater tendency than others to use analogies in our thinking and in our papers, I am glad that my time for presentation was limited, for I had intended to refer to acquired hypogammaglobulinemia as an "immunological paresis"; to extreme hypergammaglobulinemia associated with diffuse liver disease as "immunological epilepsy"; to hypergammaglobulinemia associated with extreme susceptibility to infection as "immunological cerebellar disease"; and perhaps, in some degree, to multiple myeloma as "immunological schizo-

phrenia." The latter terminology was facetiously proposed by Thomas in informal discussions of my experimental work. Such indiscretion would doubtless have induced justified apoplexy in persons inclined to a more conventional terminology.

CHRISTOPHER M. MARTIN (*National Institute of Allergy and Infectious Disease, National Institutes of Health, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.*): I should like to mention briefly a study still in progress at the National Institutes of Health, Bethesda, Md., which, although designed primarily to investigate protein synthesis rather than homotransplantation, may be of interest in connection with Harris' and Good's papers.

In June 1955, eight internal iliac lymph nodes were excised from a normal, unsensitized human donor, weighed, sliced, and transplanted to the fat of the thighs of a 64-year-old hypogammaglobulinemic woman.

Recipient and donor were then challenged with standard, half-milliliter subcutaneous doses of typhoid vaccine at a distant site. Biopsy of one of the nodes 19 days after transplantation revealed a successful initial "take," plasmacytosis, and high local antibody titers. Biopsies of the patient's indigenous nodes before and after challenge revealed neither plasma cells nor antibody.

Immunochemical studies and appropriate mathematical analysis indicate that, at the peak of the primary immune response, the transplanted nodes synthesized antibody at the rate of 4 to 8 mg. per gram wet weight of tissue per day and, at the peak of the secondary response, 8 to 15. These very brisk rates of protein synthesis are quite comparable to those at which human and animal endocrine, exocrine, and liver tissues can synthesize protein hormones, digestive enzymes, and plasma proteins *in vitro*.

Persistence of systemic antibody titers and responsiveness to later challenges indicated full function of the transplanted nodes for 130 days, and partial, diminishing function to 160 days.

Although donor and recipient were of different blood types, the recipient developed neither an anemia nor a positive Coombs test. Whether or not it is relevant, however, the recipient developed a severe neutropenia, which lasted from day 50 to day 220.

The latter point and other clinical considerations—principally the risk of transmitting the agent of serum hepatitis—suggest that lymph-node homotransplantation entails considerable risks for the recipient and that, although clearly feasible as an experimental tour-de-force, it is at present of questionable value in the long-term treatment of acquired hypogammaglobulinemia.

C. ANDREW L. BASSETT* (*Naval Medical Research Institute and Tissue Bank, Naval Medical School, Bethesda, Md.*): During the course of a study on the characteristics of human skin cells in long-term tissue culture, a method was sought for the selective inhibition of the cellular components.^{1, 2} An anti-human skin-immune globulin was developed in rabbits by immunization with an antigen derived from tissue cultures of the cellular elements of adult human split-thickness skin. The rabbit-immune globulin demonstrated profound

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and irreversible cytopathogenic effects when administered to washed cultures of cells originally constituting the antigen. Further, this globulin showed extensive cytotoxic activity against cultures of cells of ectodermal and mesodermal origin from the adult or fetal human. Little or no effect was obtained *in vitro* against cells arising from C3H mouse tissues, including skin tissue.

Originally it was found, by trial and error, that the presence of chicken plasma, as a cell substrate in culture flasks, obliterated the cytotoxic manifestations of the immune globulin completely. This inhibition could be overcome by markedly increasing the dosage of globulin. Additional investigation showed that washed preparations of susceptible cells, under a perforated cellophane substrate, were completely protected against previously lethal doses of globulin by substituting as diluent frozen, pooled, human serum for saline. Increasing the concentration of immune globulin ultimately yielded a "break-through effect." Boiling the serum removed its inhibitory activity on globulin cytopathogenicity.³ It is proposed, therefore, that some of the conflicting results previously obtained in the *in vitro* assay of antisera cytopathogenicity may represent a dosage phenomenon stemming from the presence of plasma and/or serum in the experimental system.

References

1. BASSETT, C. A. L., V. J. EVANS, D. H. CAMPBELL & W. R. EARLE. 1955. The characteristics and potentials of long-term cultures of human skin. Naval Med. Research Inst. Proj. Rept. NM-007-081.10.11, Bethesda, Md.
2. BASSETT, C. A. L., V. J. EVANS & W. R. EARLE. 1956. Characteristics and potentials of long-term cultures of human skin. Plastic & Reconstr. Surg. 17: 421.
3. BASSETT, C. A. L., D. H. CAMPBELL, V. J. EVANS & W. R. EARLE. 1956. The cytotoxic activity of rabbit immune globulin prepared from tissues of human skin and whole human placenta. Submitted for publication.

THE LEVEL OF GAMMA GLOBULIN POSTOPERATIVELY AFTER PERFORATING KERATOPLASTIES: A PRELIMINARY REPORT ON 14 CASES

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According to the opinion generally held today and from the biological point of view, the success of every tissue graft depends primarily on immunity processes that develop in the organism of the recipient as a reaction to the implanted graft. This rule is also held to apply to a corneal graft, even though the exceptional status of the anterior chamber of the eye, which is lined by the cornea and has no blood vessels, is generally known, at least as regards the development of immunity processes.

Since the majority of circulating antibodies are contained in the gamma globulin fraction of the blood serum protein, electrophoretic examination would appear to be an acceptable method for the global study of these processes.

This form of examination, one of a complex of examination methods that are carried out in our cases, has so far been applied to 14 patients, in whom partial perforation keratoplasty was performed for keratoconus, leukoma of varying origins, maculae of varying origins, and grafts that had become opaque after transplantation. Corneas that had been preserved on the bulbi in a moist chamber at $+4^{\circ}$ C. for 1 to 3 days and that had been enucleated in donors who complied with the usual conditions were used as grafts. As the control group, 10 patients who had undergone cataract extraction, in most cases by extracapsulation, were selected.

Blood was always collected for examination at least once before the operation and on the actual day of surgery. In addition, blood was usually collected several times before the operation at intervals of several days, most frequently at intervals of a week. In the postoperative period, the blood was collected at weekly intervals.

After centrifuging, the serum was drawn off and stored in the frozen state—at -20° C.—until the examination had been completed. This procedure was always followed when all the sera had been collected from a group of patients on whom operations had been performed.

Separation was carried out by paper electrophoresis in a moist chamber at a potential gradient of seven and one-half volts per centimeter and an intensity of 0.18 milliamperes per centimeter of the width of the paper with a veronal-citrate buffer of pH 8.6. The dry papers were stained with bromphenol blue by the usual method, and the intensity of the staining was determined photometrically with a blue filter after washing in a solution of 8 per cent citric acid in methanol. Extinction of the individual fractions was estimated photometrically, and the results were expressed in percentage.

Patients who had received a corneal graft were divided according to bio-microscopic examination into four groups. The first of these groups included four patients with keratoconus, in whom the cornea was free of blood vessels. The second included six patients with vascularization of the cornea. The third

consisted of one patient in whom retransplantation had been carried out after one year and in whom the cornea was also infiltrated with blood vessels. The fourth group comprised three patients in whom vascularization was not demonstrated in the critical period but could not be excluded with complete certainty.

In the first group, the values obtained were in the region of normal values in three cases. In one patient, an increase in the gamma-globulin values was found.

In the second group, one case was omitted from the evaluation because the operation was carried out at a time when the gamma globulin was at the peak of its ascent. In the clinical picture, an equivalent was found only in a rise in temperature repeated several times, without any other signs. After the operation, the curve gradually fell. An interesting observation was made in the same case. After the operated patient had been discharged with a clear graft, he returned six months later because of *maladie du greffon*. Before starting cortisone, blood was again collected for examination. The gamma-globulin values obtained from this subject and in two other samples of blood collected at weekly intervals were found to be practically the same as before discharge. In the remaining five patients operated on, increased values were found in one; in the other four, the values ranged within the limits of the normal.

In the patient in whom retransplantation had been carried out, the values found did not deviate from the normal.

The second and third groups were divided simply for reasons of method and, in the present evaluation, it will be better to take them together. In these six patients with markedly vascularized corneas, the curve rose above normal values in only one, whereas in five the values remained within normal limits, a fact which is worthy of note.

In the last group of patients, in whom vascularization in the critical period was not demonstrated but in whom it could not be excluded with complete certainty on the basis of the examinations carried out, an increase in gamma globulin occurred in two cases; in one the values remained within normal limits.

Since all the patients were in good general health, it can be assumed that the organism of each was capable of an immunity reaction. An increase in values occurred between the second and the fourth week, which fall within the period for the development of antibodies. In two cases normal values were found in the fifth week after operation, the peak of the curve coming in the fourth week. In the two remaining cases these developments could not be observed, as the patients were discharged from institutional care four weeks after operation. In two patients whose cases were completely different and in whom grafts from the same donor had been implanted, acute necrosis of the graft, which could not have been caused by bacterial contamination, occurred on the 9th and 14th day after operation. In both these subjects unchanged values were found throughout. In one of them (retransplantation), a considerable increase in the absolute number of eosinophils was found from that period. In two other pairs, in whom a graft from a common donor had

been implanted, the curve in one pair was within normal limits while, in the second pair, there was an increase in values.

In nine patients in whom extraction of cataract was performed, all the values were within the limits of the normal scatter. In only one case, one week after extracapsular operation there occurred a considerable increase in gamma globulin, which had a clinical equivalent in a simultaneous phacoanaphylactic reaction. After the second week, the amount of gamma globulin returned to the original values.

It is certain that the values obtained do not give an accurate picture of the processes taking place in the individual groups. For this reason we have refrained, for the time being, from detailed discussion.

We should like to say in summing up only that, in 13 cases of partial perforation keratoplasty, the level of the gamma globulin did not change in nine cases and rose in four. We have not as yet found the relationship between the postoperative course, in particular the development and recession of opacity of the graft, its vascularization, the administration of drugs—for example, cortisone and antihistamines—and the final result of the operation on the one hand, and the course of the gamma-globulin curve on the other.

If substantial changes occurred in keratoplasty in 4 out of 13 cases evaluated, and if this result is compared with the result in the control group, we consider that continuation with this work, which we shall supplement still further, will enable us to provide an answer to some immunological problems in transplantation.

Finally, we should like to emphasize that the material obtained—128 electrophoreses—is quite unsuitable for a statistical evaluation or for the drawing of any conclusions. We have made a preliminary study of the subject in order to make use of the results obtained to indicate the further direction of study. We regard the present paper as no more than a preliminary communication.

THE DEMONSTRATION OF DELAYED-TYPE REACTIVITY IN CONGENITAL AGAMMAGLOBULINEMIA

By Herbert M. Porter

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Recent papers have described the inability of patients with congenital agammaglobulinemia to resist repeated infections and their failure to develop circulating antibodies after antigenic stimulation.^{1, 2, 3, 4} To date, there has been no report of the artificial induction of delayed hypersensitivity in a patient with congenital agammaglobulinemia.

This paper reports the results of immunologic studies done in a 3 $\frac{1}{2}$ -year-old male child (R.M.) with congenital agammaglobulinemia. It is of interest that a male first cousin of R.M. also suffers from this syndrome.

Immunologic Studies

Immunologic studies are summarized in TABLE 1. The antigens used in these studies were: (1) bacterial, pertussis and BCG; (2) viral, mumps and vaccinia; and (3) hapten, 2,4-dinitrofluorobenzene. In addition, cellular transfer to a tuberculin negative subject was performed to confirm the specificity of the induced tuberculin positivity.

Methods

Bacterial antigens. Pertussis immunization consisted of 3 0.5-cc. injections at 3-week intervals of pertussis toxoid. Bacterial agglutinins were sought 3 weeks after the last immunizing injection, and mouse protecting antibodies were sought at 3 months.

BCG immunization was performed by a single 0.1-cc. intradermal injection. Ten weeks later, skin testing was done with old tuberculin and the Choucrour⁵ test for tubercle bacillus polysaccharide antibody was performed.

Leukocyte transfer was accomplished by injecting the pooled white cells from capillary tube centrifugates of sterile heparinized blood. The capillary tubes were snapped off at the red cell-white cell junction. The plasma leukocyte layers were pooled and centrifuged. After the supernatant plasma was removed, the white cells were washed in normal saline twice and resuspended in 0.5 cc. of normal saline prior to intradermal injection.

As controls, plasma and plasma-red cell suspension from R.M. were tested separately in another tuberculin negative subject who subsequently also received leukocytes from R.M. The test subject for cellular transfer was a tuberculin-negative premature infant with a severe cerebral anomaly and the physiologically low gamma globulin of 0.86 Kunkel units.

Viral antigens. Mumps immunization consisted of 2 injections of 1.0 cc. each of mumps vaccine (Lederle) with a 10-day interval between injections. Skin testing was done with chick allantoic fluid antigen and a heated control. Complement fixation was done by the New York City Department of Health.

Vaccination was done in the skin of the deltoid area by the multiple-puncture technique at 19 and 23 months of age. The local skin response was noted.

TABLE 1
IMMUNOLOGIC STUDIES

Antigen	Response studied	Result
<i>Bacterial</i>		
1. Pertussis	1. Bacterial agglutinins 2. Mouse protecting antibodies	1. Negative 2. Negative
2. BCG	1. Polysaccharide antibody 2. Tuberculin testing 3. Cellular transfer	1. Negative 2. Delayed hypersensitivity 3. Delayed hypersensitivity
<i>Viral</i>		
1. Mumps	1. Skin test 2. Complement fixation	1. Negative 2. Negative
2. Vaccinia	1. Skin response	1. Accelerated
<i>Hapten</i>		
1. 2,4-DNFB*	1. Skin response	1. Delayed hypersensitivity

* 2,4-dinitrofluorobenzene.

Hapten. A quantity of 2,4-dinitrofluorobenzene^{6,7} was applied on a patch of filter paper moistened with this substance and taped to the interscapular area for 48 hours. Skin testing was similarly done 10 days later at a lower site on the back with a chemical homologue 2,4-dinitrochlorobenzene. The latter substance was chosen as a test to eliminate the possibility of a serious allergic reaction. The possibility that 2,4-dinitrochlorobenzene might cause a local chemical irritation in the concentration used (0.01 M) was ruled out by skin-testing 6 normal controls with this substance applied as a patch. In no case did erythema or induration occur.

Results

Pertussis agglutinins and mouse protecting antibodies were not found.

Ten weeks after BCG immunization, an abscess from which live organisms having the morphological and growth characteristics of BCG were isolated, developed in the site of inoculation. These organisms were avirulent for the guinea pig. The test subject became tuberculin-positive. He was tuberculin-negative up to 1:100 dilution O.T. 1 week before immunization. Polysaccharide antibody could not be demonstrated. The tuberculin negative test and control subjects to whom leukocytes from R.M. were passively transferred developed transient skin hypersensitivity to tuberculin at the site of cellular transfer. The control remained tuberculin-negative when given intradermal injections of plasma and a plasma-red cell suspension from R.M.

The mumps skin test was negative, and there were no complement-fixing antibodies.

A typical accelerated response followed the second vaccination at 23 months of age.

Dinitrofluorobenzene caused an initial vesicular eruption beneath the sensitizing patch. The vesicles coalesced and dried, forming an eschar. Application of a test patch with dinitrochlorobenzene produced an erythematous, indurated, elevated area precisely the size and shape of the test patch, signifying a delayed hypersensitivity to dinitrofluorobenzene-protein conjugate.

Discussion

The data reveal that circulating antibodies of the gamma-globulin variety were not made, but there is no defect in the mechanism responsible for production of delayed hypersensitivity. The accelerated response following vaccination is believed, however, to signify the presence of immune substance resulting from a previous infection (primary "take").⁸ This seeming paradox may be explained in several ways. There may have been gamma-globulin antibody present in amounts sufficient to have a protective effect, but in quantities too minute to be measured by current techniques.

A second possibility may lie in the development of protective antibodies of a noncirculating, that is, tissue-fixed, variety. This explanation suggests the co-existence within or on the cell of both a protective and allergic (delayed hypersensitivity) mechanism similar to the condition that prevails in the serum. This concept may be more readily acceptable if one considers that viruses are obligate intracellular parasites. There is, however, no real evidence to support this theory.

A third possibility is that the delayed hypersensitivity actually plays a role in offering the host limited protection. For example, the tubercle bacillus in the center of an area of caseation necrosis is destroyed.⁹ In an analogous manner, the virus of vaccinia or other viruses that cause exanthems may be prevented from proliferating. On the other hand, viruses that do not cause skin hypersensitivity would not be prevented from proliferating unless, of course, tissues other than skin and leukocytes were individually capable of developing delayed hypersensitivity. Patients with agammaglobulinemia would appear to resist repeated recognized infections with measles and chicken pox, though not necessarily mumps, for only a small percentage of patients with mumps ever develop a positive skin test.

Finally, it must be remembered that, as rubella has been shown to occur without exanthem, it is possible that other virus infections, such as measles, may occur without recognized exanthem. Needless to say, most of the "grippelike" syndromes seen in pediatric and general practice, that is, "the virus," remain without an exact etiological diagnosis. Substances such as lysozyme, complement, and properdin,¹⁰ no doubt, also play an important role.

Summary

A patient with congenital agammaglobulinemia was shown to be capable of producing delayed hypersensitivity in the absence of circulating gamma-globulin antibodies. The independence of the tuberculin response on gamma globulin is affirmed.

It is felt that the development of delayed hypersensitivity protects the patient to a limited degree from repeated infections with measles, chicken pox, rubella, roseola and, possibly, mumps.

References

1. BRUTON, O. C. 1952. Agammaglobulinemia. *Pediatrics*. **9**: 722.
2. BRUTON, O. C., L. APT, D. GITLIN & C. A. JANEWAY. 1952. Absence of serum gamma globulin. *Am. J. Diseases Children*. **84**: 632.

3. HAYLES, A. B., G. B. STICKLER & R. F. MCKENZIE. 1954. Decrease in serum gamma globulin. Report of three cases. *Pediatrics*. **14**: 449.
4. JEAN, R. 1953. Isolated hypo or agammaglobulinemia in children. *Presse méd.* **61**: 828.
5. CHOUCROUN, N. 1947. Tubercle bacillus antigens. Biological properties of two substances isolated from paraffin oil extract of dead tubercle bacilli. *Am. Rev. Tuberc.* **56**: 203.
6. LANDSTEINER, K. & M. W. CHASE. 1937. Studies on sensitization of animals with simple chemical compounds: anaphylaxis induced by picryl chloride and 2,4 dinitrochlorobenzene. *J. Exptl. Med.* **66**(3): 337.
7. EISEN, H., I. ORRIS & S. BEITMAN. 1952. Elicitation of delayed allergic skin reactions with haptens: the dependence of elicitation on hapten combination with protein. *J. Exptl. Med.* **95**(5): 473.
8. RIVERS, J. B. 1948. *Viral and Rickettsial Infections of Man*. J. B. Lippincott Co. Philadelphia, Pa.
9. RAFFEL, S. 1953. *Immunity, Hypersensitivity, Serology*. Appleton-Century-Crofts. New York, N. Y.
10. PILLEMER, L., L. BLUM, I. LEPOW, O. ROSS, E. TODD & A. WARDLOW. 1954. The properdin system and immunity. I. Demonstration and isolation of a new serum protein, properdin, and its role in immune phenomena. *Science*. **120**: 279.

Discussion of the Paper

GUY VOISIN (*Hôpital Saint-Antoine, Paris, France*): I believe it is particularly important to study the nature of the hypersensitivity that is thought to be related to the rejection of homografts.

Several authors believe that we deal with a delayed or bacterial type of hypersensitivity. In his extremely interesting presentation Sherwood Lawrence summarized about all the arguments in favor of this point of view. The main ones are: the usual delay of the rejection of the homograft in about 10 to 12 days; the accelerated rejection of a second graft of the same donor to the same recipient in 5 to 7 days (second-set phenomenon); the dosage factor; the necessity of a living or at least intact and complex antigen, be it a bacterium or a living homograft found in hypersensitivity in homograft as well as with bacteria; the impossibility of passive transfer of this hypersensitivity by means of the serum of the recipient previously sensitized by a homograft of the same donor; and the possibility of transfer of this hypersensitivity by means of cells of the same recipient (Billingham, Brent, and Medawar).

But some of these arguments favor this phenomenon of hypersensitivity without defining its type, and some of them never proceed beyond the stage of discussion or interpretation. For instance:

(1) The fact that a homograft is rejected tardily, even in a second-set situation, does not necessarily prove the existence of the delayed type of hypersensitivity, but may stress only the necessity of an adequate vascularization for graft rejection.

(2) At present, intact living cells used as sensitizing antigen seem to be essential for the "homograft type" of hypersensitivity only. On the other hand, it is possible to produce the tuberculin type of hypersensitivity, not only with killed bacilli, but also with chemical fractions of the tubercle bacillus.

(3) Billingham, Brent, and Medawar have been able to show that it is possible to suppress acquired tolerance toward homografts of a donor by passive transfer of cells of a nontolerant recipient who has previously received a graft

from the same donor. They obtained the same result, however, by transferring passively cells of animals that had not received a graft.

On the other hand, there are certain observations in the homograft type of hypersensitivity in which at least a contributory effect has been attributed to the serum. The main ones are: (1) the fact that homografts are tolerated by patients with agammaglobulinemia; and (2) the unfavorable effect of the serum of the recipient on the cells of the donor (the *in vitro* effect of Billingham and the *in toto* effect of Woodruff).

The preceding facts therefore indicate that, at the present time at least, it may be wise to speak of hypersensitivity in connection with the rejection of homograft as of a particular and complex type in which the cells, as well as the serum of the recipient, play their roles and which cannot be considered as belonging to a simple and already known type of hypersensitivity. I suggest that this be called the "homograft" type of hypersensitivity.

Part III. Embryonal, Adult, and Tumor Tissue Homotransplants

THE POSSIBILITIES OF BREPHOPLASTIC TRANSPLANTS

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Positive results have been obtained, in general, with transplantations in adult mammals in 3 cases: autotransplantations, transplantations of dead tissues, and transplantations of tissues still in the embryonic state. The latter, consisting of cells that still possess a developmental potential and whose specificity and antigenicity appear weak, seem able to adapt themselves to their new environment, to become incorporated in it, and to adopt its chemical pattern.

My associates and I have systematized such operations, after making them in numerous tissues and organs. In 1934 we devised the term "brephoplastic transplantation" from the Greek words *brephos*, "newborn," and *plassein*, "to model," applying it to functional and durable transplantations of tissues from embryonic or newborn animals to young or adult specimens of the same species. Thus these operations are designated as a special case of homotransplantation.

A survey of the literature shows that, while transplantations of adult tissues in mammals have occasionally achieved positive results, most of the success has been obtained with tissues that, when grafted, were still in an embryonic state. It seems to us that, in this respect, we have a very close parallelism between transplantation and tissue culture. Here also growth has been obtained essentially with embryonic tissues, while adult tissues can be cultured only in special cases.

The loss of the embryonic transplantation potential is well shown by the following experiments, which we described in 1947. Thirty-six thyroid lobes, taken from newborn albino mice and from mice 5, 9, 14, 21, 31, 40, and 43 days old, of the same strain, were grafted in the anterior chamber of the eye of adult mice. In each series these lobes were studied histologically, at intervals between 4 and 38 days after their transplantation.

In spite of the presence of a normal thyroid, the lobes from newborn mice, still embryonic in appearance, lost their basophily, acquired numerous colloid follicles, and took on the aspect of normal thyroid tissue of the same age. When the transplant was from very young mice (5 days old), the host tissues showed a reaction that was insufficient to cause the graft's degradation. When the implanted lobe no longer had any embryonic characteristics (onward from 9 days of age), however, the host reaction caused its degradation, with a rapidity that increased with the age of the graft.

Thus a complete adaptation of a thyroid graft is possible, even within the same strain of albino mice, only if the graft is transplanted while still in the embryonic state.

At first most of our brephoplastic transplantations were made with laboratory rodents of the same or closely related strains, thus avoiding the reactions be-

tween tissues completely foreign to each other. Nevertheless, the fact remains that even here, while one obtains positive results when the transplanted tissues are still embryonic or very young, they fail to "take" when these tissues originate from older animals. The additional facts that we shall now bring to your attention prove that consanguinity is not indispensable in brephoplasty.

Together with Marie-Jeanne Thillard, we experimented with 2 distinct pure strains of rabbits: 1 albino, the other fully pigmented. The 2 strains came from separate and distant regions of France. We first implanted (FIGURES 1 and 2) in the anterior chamber of the eye of both strains a thyroid lobe, an adrenal gland, or a hypophysis taken from a newborn rabbit of the other strain. The animals were sacrificed 24 to 42 days after the transplantation.

Histological examination of the host eyes showed that in almost all cases (7 out of 9) the endocrine transplant was fixed on the iris and was well-vascularized, with perfectly developed tissues. Thus numerous mitoses were found in certain adrenal glands; the parathyroid tissue was developed and distinct from the thyroid follicles; and anterior lobe tissue of the hypophysis was well differentiated. The size of the transplants, however, was much smaller than that which would have been attained *in situ*, and peripheral lymphocytes were found in several cases.

Having shown, in 1948, that a strong percentage (8 out of 15) of thyroid lobes from newborn mice still "take" in the anterior chamber of the eye of adults of the same species after having been kept 49 to 65 hours in physiological saline at 4° to 6° C., we applied a similar technique to the newborn rabbit endocrine glands. These were placed, almost always whole, on Wolff and Haffen's (1951) substratum of agar-agar, 1 per cent in Ringer's solution, and

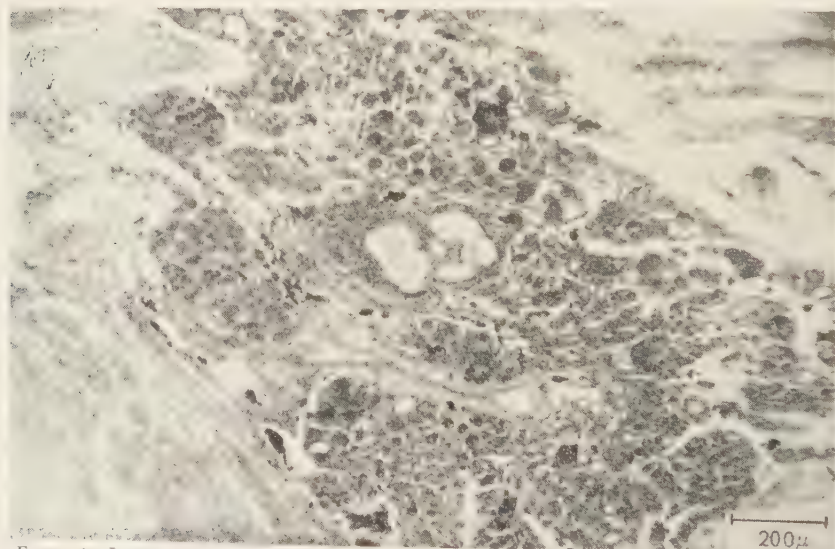


FIGURE 1. Interstrain intraocular transplantation of a noncultured thyroid 28 days after its implantation. The thyroid from a newborn pigmented rabbit is in the eye of an albino young rabbit. Clusters of lymphocytes (XII-49).

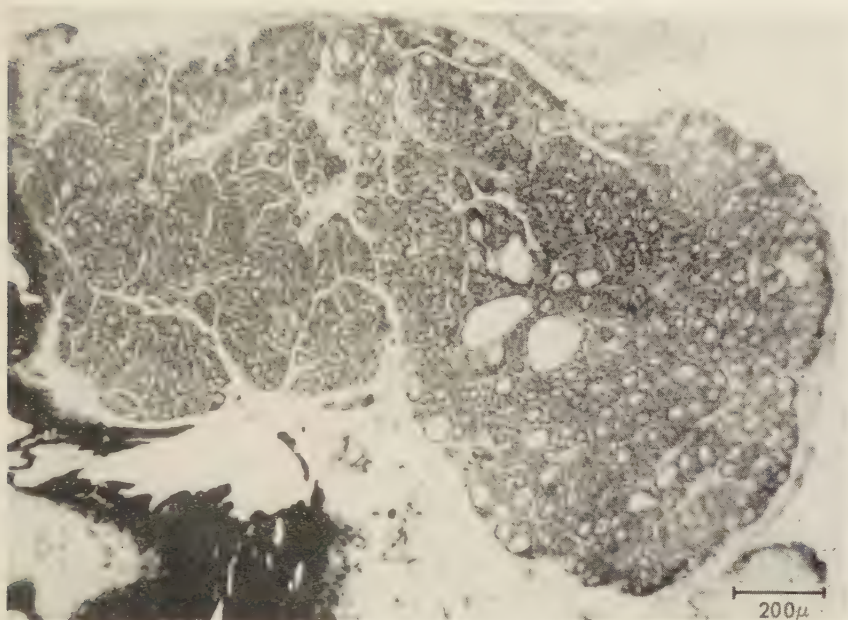


FIGURE 2. Interstrain intraocular transplantation of a cultured thyroid 32 days after its implantation. The thyroid from a newborn albino rabbit is in the eye of a pigmented young rabbit. Mitoses, no lymphocytes (IX-28).

bathed in equal parts of Ringer's solution and serum of the future host. Kept at 37°C . under these conditions, almost all the nuclei became pycnotic. But at 5° to 6° almost all the nuclei were perfectly preserved after 2 to 3 days.

When glands, thus kept at a low temperature in the serum of the host of another strain, are placed in the latter's anterior eye chamber, a positive result is obtained in more than half of the cases. Thus in 1 series, out of 4 thyroid lobes, 3 adrenal glands, and a hypophysis from a pigmented strain that we recovered from 14 to 90 days after their implantation in an albino's eye, we found histologically 1 thyroid, the cortex of 1 adrenal gland, 2 other particularly well-developed adrenals with both the cortex and the medulla, and a pars anterior of the hypophysis. In an opposite series, out of 4 thyroid lobes and 2 adrenals from a newborn albino implanted in a pigmented eye, we found, 14 to 32 days after their transplantation, 3 well-developed thyroid lobes, with their parathyroids. The cells of all these transplants were in excellent shape, and there were very few lymphocytes present. It is curious to note that results are not better when a gland that has been cultured at a low temperature is implanted in the eye of a rabbit of its own albino strain.

We can conclude from these results, first, that brephoplastic transplantations are possible between 2 pure and distinct strains of rabbits. Second, the conservation of grafts in the serum of the future host is possible for a few days at a low temperature, but not at the normal temperature of 37°C . This is probably due to the fact that the glands of newborn rabbits are too large to be

cultured under the latter condition. Third, the percentage of "takes" among such cultured glands is smaller than among glands grafted without previous culture. Those cultured transplants that do take, however, show a better development, and there is less reaction on the part of the host of another strain than in the case of uncultured grafts.

The anterior chamber of the eye is not a site that lends itself to practical applications for transplantations in man. We therefore experimented with various sites in animals. We tried the tibial marrow; the lumen of the jugular vein; the rectus abdominis muscle (FIGURE 3); artificial edematous swellings obtained by injecting Ringer's solution intradermally; a subcutaneous region in the ear; and the subcapsular space in the kidney (FIGURE 4), using as transplants thyroid lobes and adrenals from newborn rabbits of the other strain.

Twenty intratibial cases were all negative. Of 13 intrajugular grafts we found only 1 thyroid, 21 days after its transplantation. Likewise, 8 intradermal and 7 subcutaneous grafts were all negative.

Results were better with intramuscular transplants. From 20 intramuscular grafts we obtained, after 30 days, an adrenal with its cortex perfectly preserved and with medullary zones; 3 to 4 weeks after their implantation, we obtained 3 other adrenals and 3 thyroid lobes, with characteristic histological structures separated by a certain amount of connective-tissue infiltration.

The best results were obtained with adrenals transplanted under the kidney capsule. Of 13 rabbits thus grafted there was only 1 negative case. With noncultured adrenals we occasionally found some lymphocytes around the

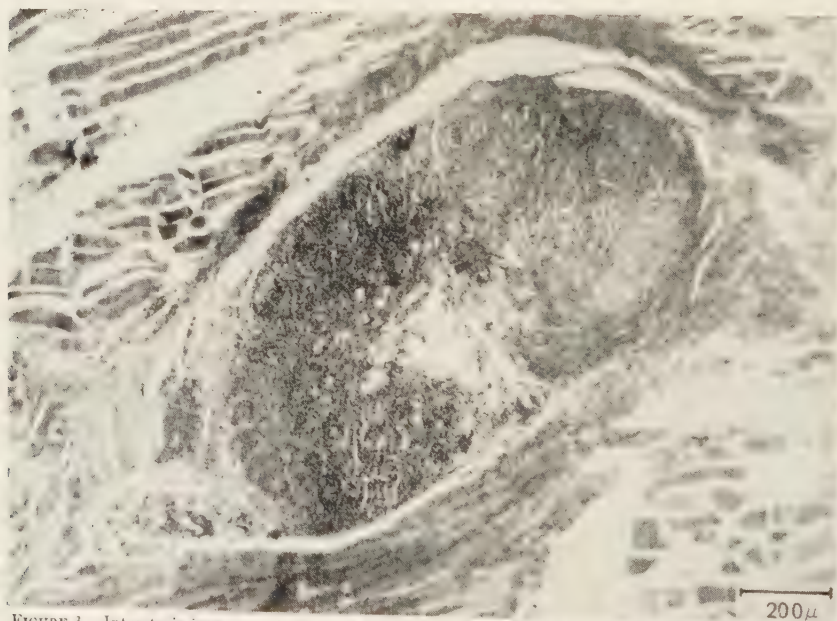


FIGURE 3. Interstrain intramuscular transplantation of a noncultured adrenal 30 days after its implantation. The adrenal from a newborn albino rabbit is in the rectus abdominis muscle of a pigmented young rabbit. Well-developed cortex on the right, medullary zone in the center, migratory cells on the left (XIV-69).

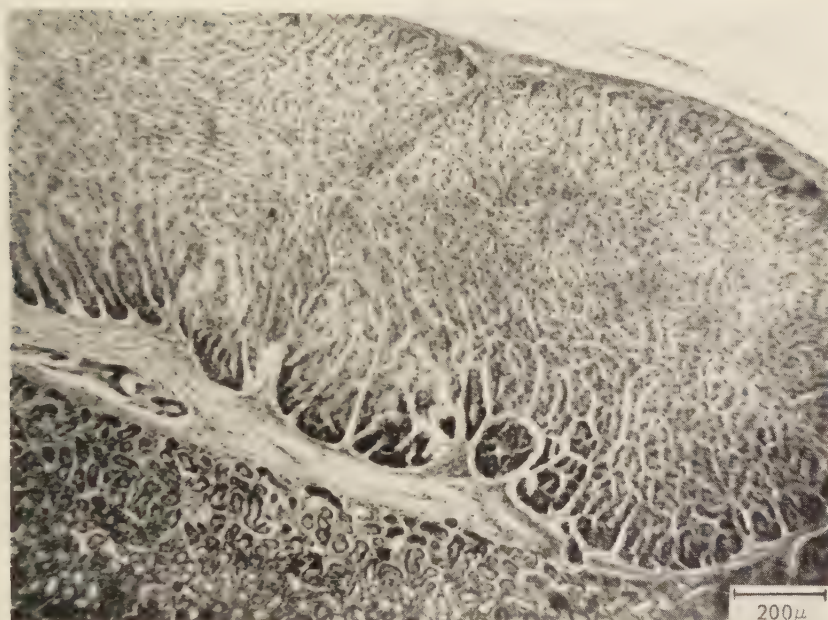


FIGURE 4. Interstrain transplantation in the subcapsular space of the kidney of a noncultured adrenal 72 days after its implantation. The adrenal from a newborn albino rabbit is grafted in a pigmented host. Well-developed cortical zone without migratory cells (XXII-C12).

cortical tissue up to 72 days after the graft. The cortical tissue of 2 adrenals cultured in the host's serum, however, as described above, was perfectly free of lymphocytes. Vascularization was always good.

Four other adrenals were irradiated, previous to their culture, with 70 kv. and a dosage of 55 or 113 r. In all 4 cases we found, after 31 days, a large mass of cortical tissue in excellent shape and without any lymphocytes. Another adrenal was cultured at 5° C. for 2 days in aqueous humor from an ox's eye. After 31 days in the subcapsular space of the kidney, its cortex was in good shape and without lymphocytes, but of small size.

We may draw the following conclusions:

(1) In brephoplastic interstrain transplantation of adrenals in the rabbit, the anterior chamber of the eye is by far the best site. The grafts persist there in good shape, but do not grow. The subcapsular space of the kidney permits a better development of the adrenal's cortex while, in the rectus abdominis muscle, one obtains a certain percentage of successful grafts.

(2) Culture at a low temperature of endocrine grafts in the host's serum often avoids the latter's lymphocytic reactions.

(3) Irradiation with 70 kv. and a dosage up to 113 r also give a good development of the cultured glands without any lymphocytic infiltration.

Another aspect of brephoplastic grafts is their functional value. We have long since demonstrated this aspect in the case of thyroids and parathyroids grafted in the anterior chamber of the eye (1932), subcutaneously (1934)

[later confirmed by Salmon and Severinghaus (1936)], and in the tibial marrow (1939) of albino rats of the same strain. Such functional grafts grow and retain a perfect histological aspect and vascularization for nearly 17 months in the case of some of our subcutaneous transplants (1936). We have given further examples concerning other endocrine glands in our book *La Greffe* (1952).

Since then, Deanesly (1954) has shown that rat prepuberal ovaries and testicles can be frozen to -79° C. in 15 per cent glycerol-saline and that, after being thawed, they can be implanted subcutaneously and regain their growth, their differentiation, and their functions—ovogenesis or spermatogenesis.

As concerns the hypophysis, we showed in a very few cases (1935, 1937) that it "takes" perfectly when grafted brephoplastically in the anterior chamber of the eye of young hypophysectomized rats, and that it has a functional value. Greene (1955) confirmed these results with adult pituitary tissue in the eyes of hypophysectomized rats, thus preventing the occurrence of signs of pituitary deficiency. Here the atrophy of gonads characteristic of hypophysectomized rats does not occur, and fertile matings have been obtained.

In order to study the local effect of brephoplastic grafts of hypophysis on the gonads it is necessary to hypophysectomize the host. This was done by Gardner and Hill in 1935 and 1936. These investigators found that a hypophysis from a litter mate, grafted in the testicle, maintains its normal state or reestablishes spermatogenesis in a testicle atrophied after hypophysectomy.

More recently Aron and Petrovic (1953, 1955) and Petrovic (1954) have obtained intratesticular grafts of adult hypophyseal tissue in guinea pigs. The local action in such nonhypophysectomized animals is seen as a hypertrophy of the interstitial tissue and an arrest of spermatogenesis.

We ourselves (May, 1955) introduced into this question 2 new approaches. The first is a new technique of total hypophysectomy in prepuberal mice. The second is the use of 1 testicle as a control for the other.

The cranium of young mice of 7 to 12 gm. being incompletely ossified, we (May, 1953) delimited as a small square the bony region that corresponds to the hypophysis by puncturing it with a curved cataract needle. We then lifted, as a whole, this small square that included the occipitosphenoidal synchondrosis by means of an ophthalmological Schmitt forceps, which has small spoons built into its tips. The hypophysis came out entire in the lower spoon. With good postoperative care in an enclosure kept at 28° C. we obtained 20 to 30 per cent of hypophysectomized survivors after 1 month. The right atrophied testicle was then fixed and studied histologically. The left testicle was exposed, its tunica albuginea was raised, and we insufflated under it the hypophysis of a newborn mouse. One month later, that is, 2 months after hypophysectomy, the mouse was killed, its left testicle was fixed for histological study (FIGURE 5), and its hypophyseal region was carefully studied to detect possible hypophyseal remnants. We took into account only those cases where absolutely no such remnants were found.

The seminal tubules of the right atrophied control testicle have a diameter of

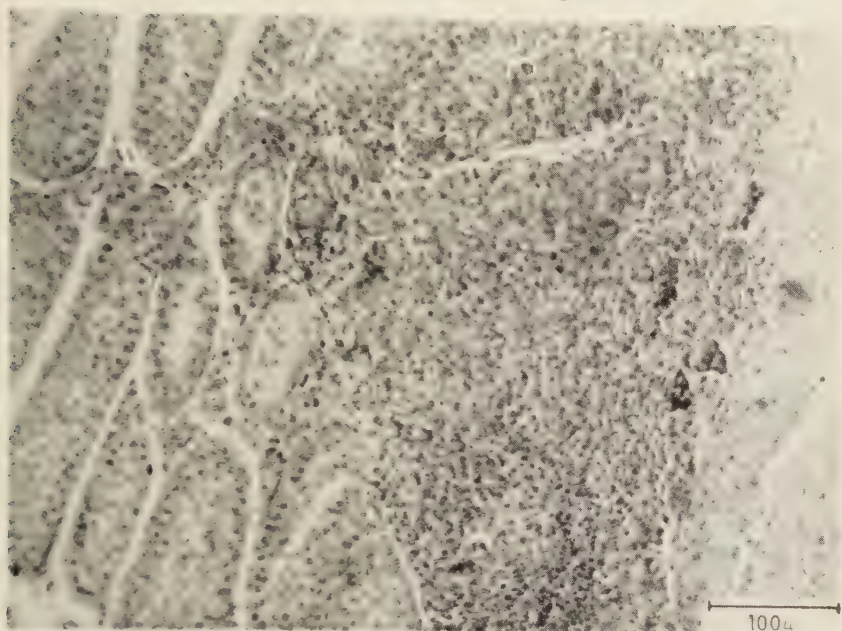


FIGURE 5. Mouse XVII-2. Hypophyseal transplant in the left testicle shown in FIGURE 6. From left to right: seminal tubules, hypophyseal graft with its cleft, tunica albuginea.

40 to 53 μ . We found in them spermatocytes, but no spermatids or spermatozoa. The interstitial tissue was reduced, very basophilic, and quite separate from the tubules (FIGURES 6 and 7).

In the left testicle we found the well-vascularized grafted hypophysis, with the various cellular types of the pars anterior, the hypophyseal cleft, and pars intermedia tissue, but no pars nervosa. The seminal tubules of such a host testicle have a diameter of 91 to 113 μ and are normally acidophile (FIGURES 6 and 7). They contain the various spermatogenic elements, with numerous spermatozoa. In 1 case we found many spermatozoa in the epididymis. The interstitial testicular tissue was well-developed and adherent to the tubules.

These results are proof of the local spermatogenic action of grafted neonatal hypophyses. Their histological aspect when fixed shows that the pars anterior has grown considerably; comparison of the 2 testicles, 1 without and 1 with the brephoplastic hypophyseal implant, leaves no doubt as to the latter's functional value.

We shall cite a final experiment, in which bone marrow was used (May and Arpiarian, 1954, 1955), that is further proof of the functional value of brephoplastic grafts. A series of 40 adult mice were X-irradiated with 70 kv. Immediately after the irradiation half of them received subcutaneous grafts of an anterior and a posterior limb, skinned and dissected free of their muscles, from newborn mice of the same strain. These bone transplants grew perfectly and remained well vascularized during several months (FIGURE 8). Sixty days after the graft the bone marrow retained a normal aspect.

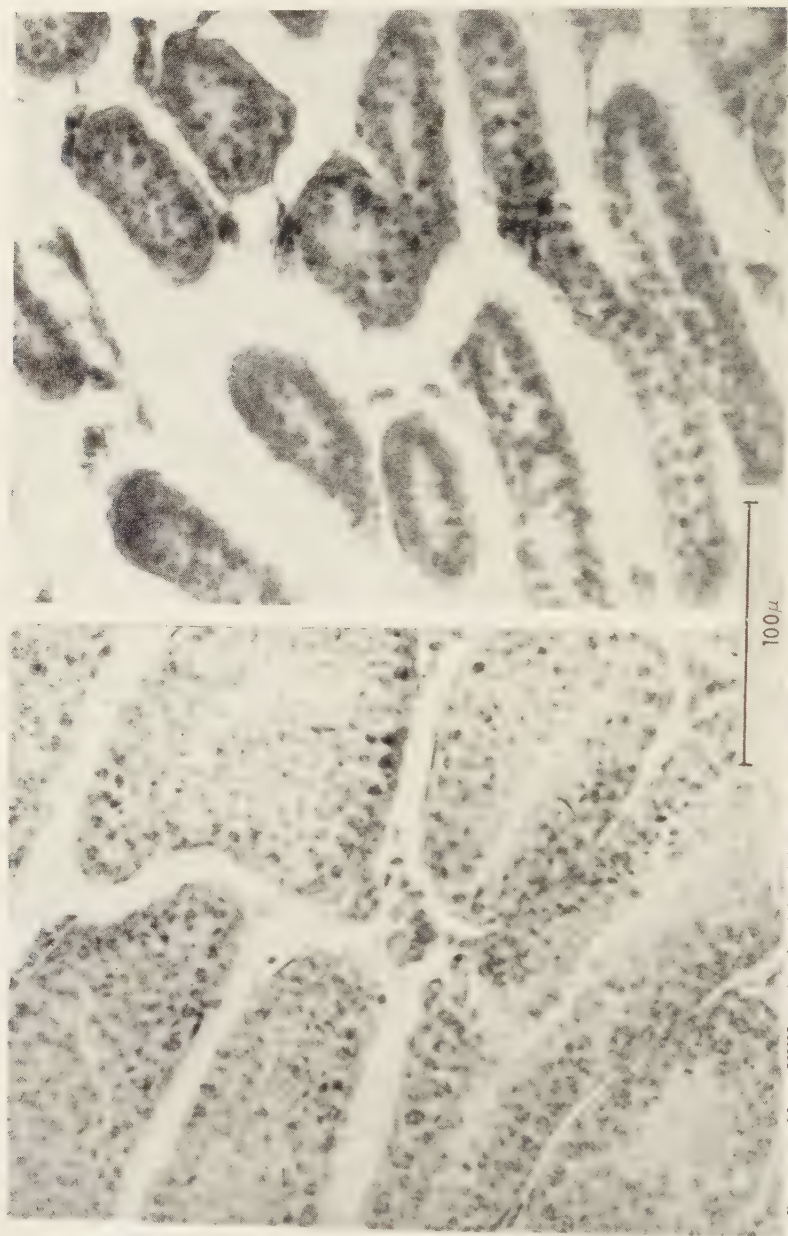


FIGURE 6. Mouse XVII-2. On the right: right testis fixed 29 days after the hypophysectomy. On the left: left testis fixed 28 days after the brephoplastic hypophyseal graft. Note the difference of diameter between the tubules of both testicles.

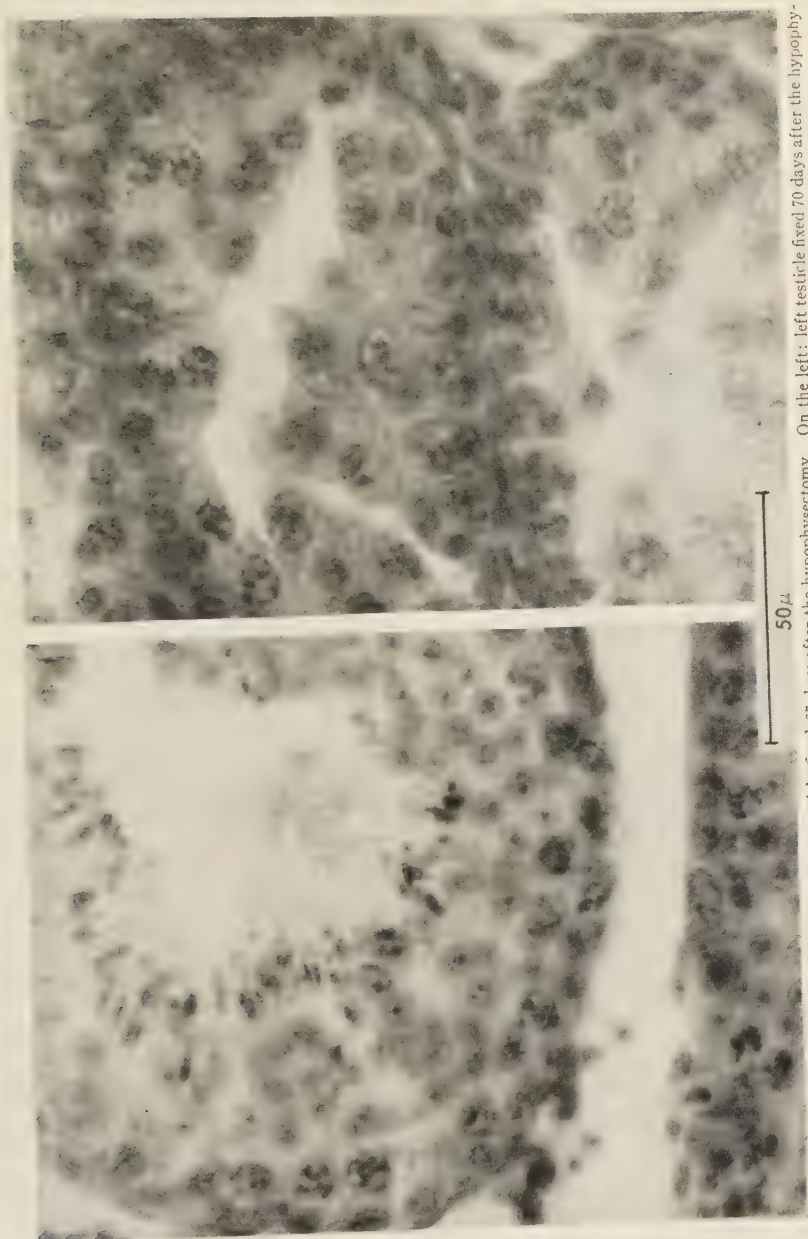


FIGURE 7. Mouse XI-1. On the right: right testicle fixed 37 days after the hypophysectomy. On the left: left testicle fixed 70 days after the hypophysectomy. Note in it the numerous normally organized spermatozoa.

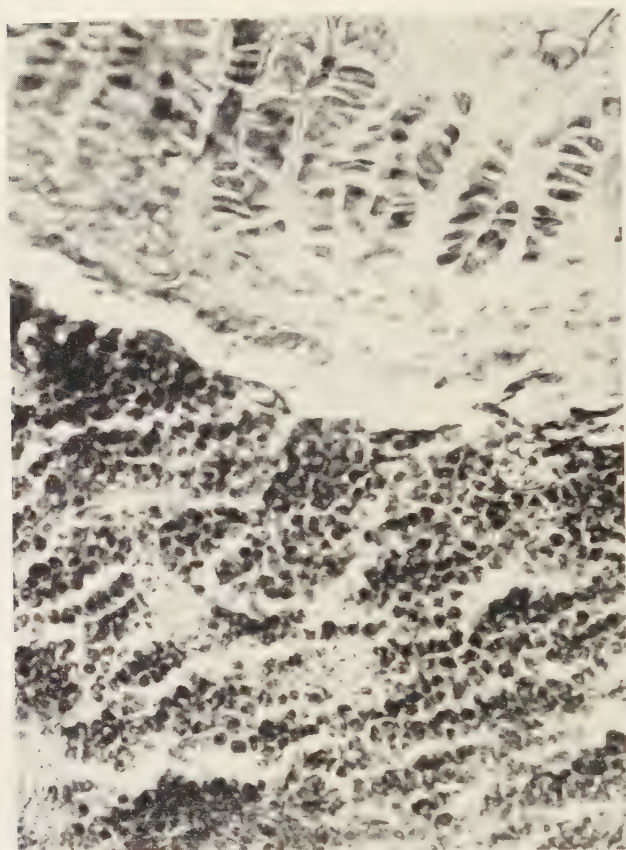


FIGURE 8. Brephoplastic graft of a limb bone under the dorsal skin of a mouse irradiated with 900 r 52 days after the irradiation and implantation ($\times 290$).

Such brephoplastic grafts had a beneficial effect against X radiation, especially at strong doses of 1100 and 1200 r. Not only was the period of survival longer, but with 1200 r we obtained 47.6 per cent of irradiated and grafted mice that lived 30 days, at which time they were usually sacrificed, against 13 per cent of mice simply irradiated. These beneficial effects also occurred with 1100 r: 50 per cent of grafted survivors against 35 per cent of nongrafted ones. With 1000 r we obtained 85 per cent of grafted survivors against 62 per cent of nongrafted ones, and with 900 r, 100 per cent of grafted surviving mice against 75 per cent of nongrafted mice (FIGURES 9 and 10).

Blood counts showed that such grafts bring about a gradual increase in the number of leukocytes in the circulating blood but no increase of erythrocytes (FIGURE 11).

We may conclude that subcutaneous brephoplastic transplants of bones containing marrow "take" perfectly and confer on irradiated mice a certain protection that accompanies an increase in the number of leukocytes.

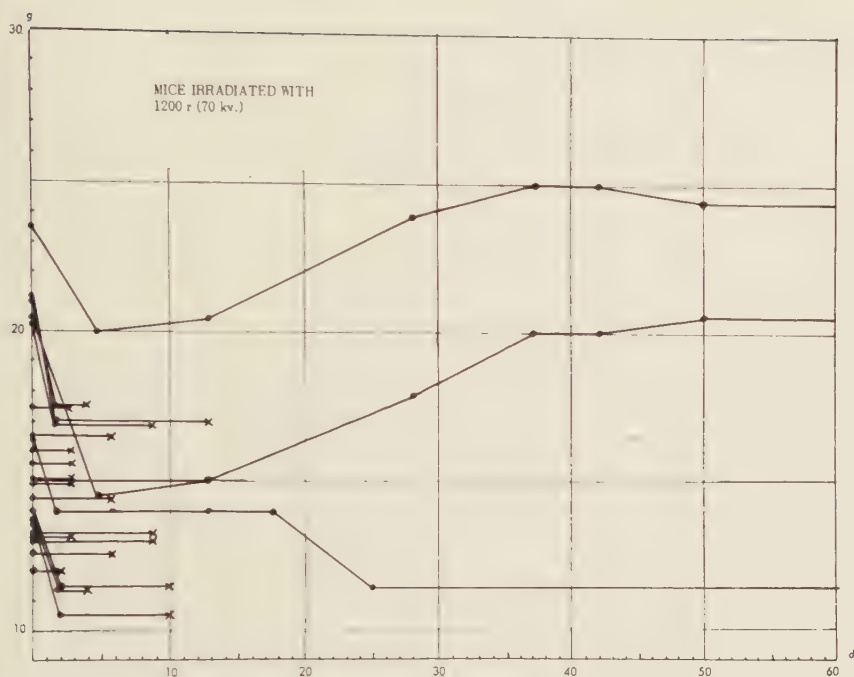


FIGURE 9. Survival curves of 21 mice that have been irradiated.

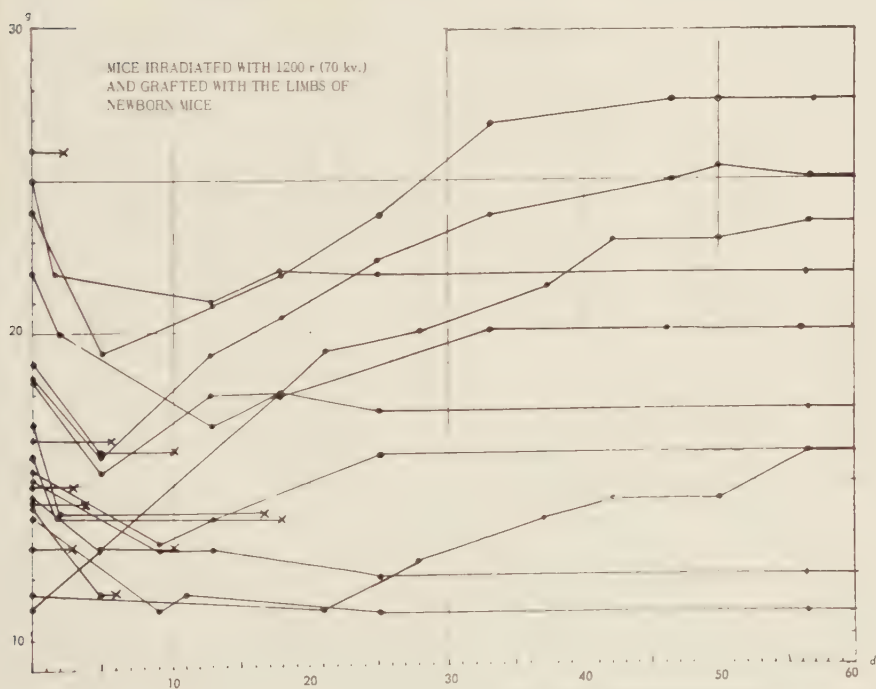


FIGURE 10. Survival curves of 20 mice that have been irradiated and grafted brephoplastically with limb bones.

Leukocytes

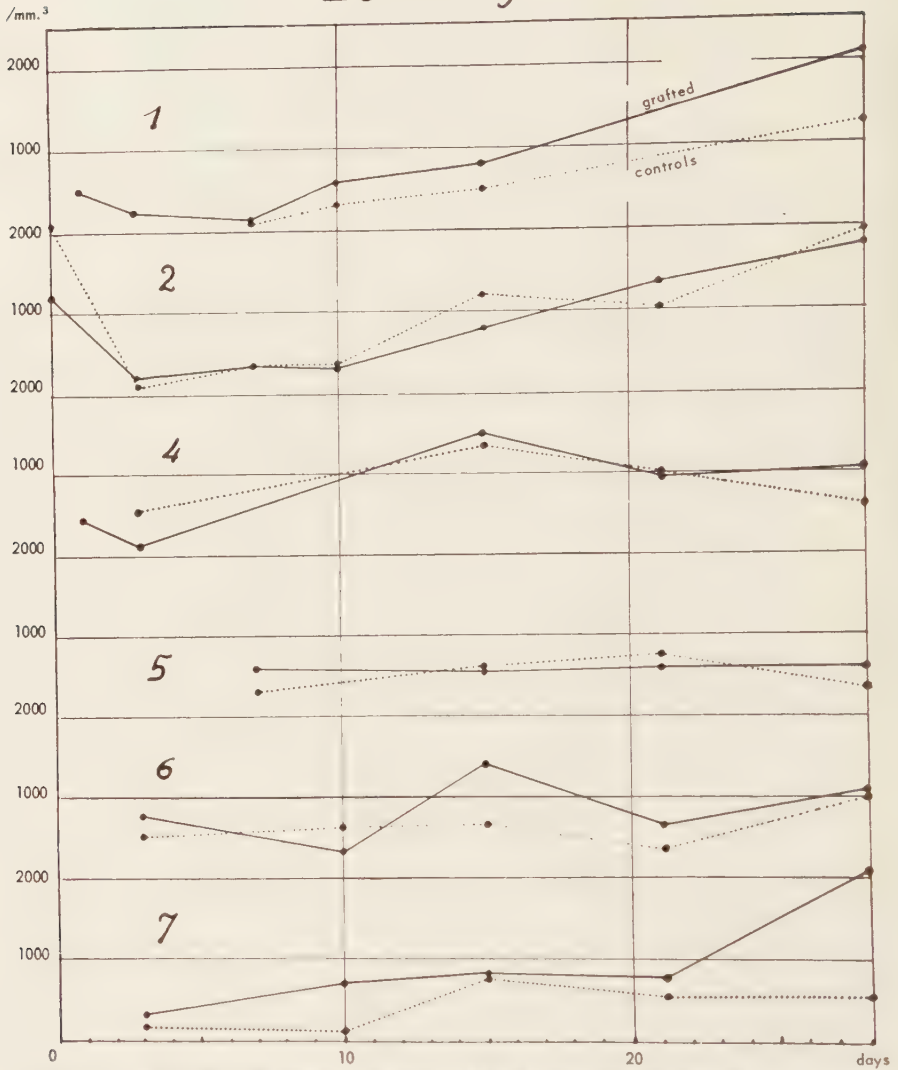


FIGURE 11. Variation in the number of leukocytes in 6 pairs of mice irradiated with 1200 r (70 kv.)—1 brephoplastically grafted with limb bones, the other not grafted.

Brephoplastic grafts have already been applied with some success as replacement therapy after parathyroid excisions in human cases (Stone, Owings, and Gey, 1935; Gaillard, 1948; Kooreman and Gaillard, 1950; Sterling and Goldsmith, 1954; and May, unpublished). These results, like the numerous positive experiments with other mammals, show that brephoplastic transplantations hold great promise and deserve further study.

References

- ARON, M., A. PETROVIC, C. WEILL & M. DEMINATTI. 1953. Homogreffes dans le testicule du cobaye. *Compt. rend.* **237**: 753-754.
- ARON, M. & A. PETROVIC. 1955. Transplantations homoplastiques et hétéroplastiques dans le testicule chez le cobaye. *Compt. rend. assoc. anat.* 41e Réunion. Gênes. **84**: 200-208.
- DEANESLY, R. 1954. Immature rat ovaries grafted after freezing and thawing. *J. Endocrinol.* **11**: 197-200.
- DEANESLY, R. 1954. Spermatogenesis and endocrine activity in grafts of frozen and thawed rat testis. *J. Endocrinol.* **11**: 201-206.
- GAILLARD, P. J. 1948. Growth, differentiation and function of explants of some endocrine glands. *Symposia. Soc. Exptl. Biol.* II. Growth. : 139-144.
- GARDNER, W. J. & R. T. HILL. 1935. Persistence of pituitary grafts in the testis of the mouse. *Proc. Soc. Exptl. Biol. Med.* **32**: 1382-1384.
- GREENE, H. S. N. 1955. Compatibility and noncompatibility. The relation of immunology to tissue homotransplantation. *Ann. N. Y. Acad. Sci.* **59**(3): 311-325.
- HILL, R. T. & W. U. GARDNER. 1936. Function of pituitary grafts in mice. *Proc. Soc. Exptl. Biol. Med.* **34**: 78-79.
- KOOREMAN, P. J. & P. J. GAILLARD. 1950. Therapeutic possibilities of grafting cultivated embryonic tissues in man. The parathyroid gland in cases of postoperative tetany. *Overg. u. Arch. Chirurg. Neerl.* **II**: 326-354.
- MAY, R. M. 1932. Action vicariante durable de la greffe intraoculaire de thyroïde de raton nouveau-né sur le développement du rat blanc éthyroïdé. *Compt. rend.* **194**: 1525-1527.
- MAY, R. M. 1934. La greffe bréphoplastique sous-cutanée de la thyroïde chez le rat. *Compt. rend.* **199**: 807-809 & *Arch. Anat. Strasbourg.* 1936. **21**: 31-64.
- MAY, R. M. 1935. La greffe bréphoplastique de l'hypophyse chez le rat. *Compt. rend. soc. biol.* **120**: 867-871.
- MAY, R. M. 1936. La durée des greffes bréphoplastiques sous-cutanées de thyroïde chez le rat. *Compt. rend.* **202**: 347-349.
- MAY, R. M. 1937. Fonctionnement sexuel normal et durable obtenu grâce à la greffe bréphoplastique de l'hypophyse chez des rates hypophysectomisées. *Compt. rend. soc. biol.* **124**: 920-922.
- MAY, R. M. 1939. Nouvelles recherches sur la greffe bréphoplastique. Thèse de médecine. : 53. Masson. Paris, France. *Arch. anat. microscop.* **35**: 147-199.
- MAY, R. M. 1947. La perte du potentiel embryonnaire de greffe au cours de la croissance de la thyroïde chez la souris. *Bull. histol. appl. et tech. microscop.* **24**(7): 135-145.
- MAY, R. M. 1948. La survie, en dehors de l'organisme de lobes de thyroïde du souriceau nouveau-né normaux et colchicinés étudiée au moyen de la greffe bréphoplastique. *Bull. histol. appl. et techn. microscop.* **25**(6): 103-111.
- MAY, R. M. 1952. La Greffe. : 301. N. R. F. (Gallimard). Paris, France.
- MAY, R. M. 1953. Nouvelle technique d'hypophysectomie applicable à de jeunes souriceaux. *Compt. rend. assoc. anat.* 40e Réunion. Bordeaux. **78**: 255-263.
- MAY, R. M. 1955. Induction de la spermiogenèse dans des testicules atrophiés de souris hypophysectomisées par la greffe bréphoplastique d'une hypophyse sous leur albuginée. *Ann. endocrinol. (Paris)*. **16**(3): 375-382.
- MAY, R. M. & N. ARPIARIAN. 1954. Effet protecteur de greffes bréphoplastiques d'os à moelle contre l'action des rayons X chez la souris. *Compt. rend.* **239**(18): 1151-1153.
- MAY, R. M. & N. ARPIARIAN. 1955. Variations des éléments sanguins concomitantes de l'effet protecteur de greffes bréphoplastiques d'os à moelle contre l'action des rayons X chez la souris. *J. physiol.* **47**(1): 238-243.
- PETROVIC, A. 1954. Recherches sur la mode d'action gonadostimulante de la préhypophyse sur le testicule chez le cobaye. Thèse doct. méd. Strasbourg. Le Nouvel Alsacien. : 76.
- SALMON, T. N. & A. E. SEVERINGHAUS. 1936. Functional auto and homoplastic thyroid grafts in the rat. *Proc. Soc. Exptl. Biol. Med.* **34**: 251-253.
- STERLING, J. A. & R. GOLDSMITH. 1954. Endocrines (parathyroid graft in human). *Transplantation Bull.* **1**: 140-141.
- STONE, H. B., J. C. OWINGS & G. O. GEY. 1935. Living grafts of thyroid and parathyroid glands. *Surg. Gynecol. Obstet.* **60**: 390-393.
- WOLFF, E. & K. HAFFEN. 1951. Sur la culture *in vitro* des glandes génitales des embryons d'oiseau: obtention de la différenciation sexuelle normale et de l'intersexualité expérimentale des gonades explantées. *Compt. rend.* **233**: 439-441.

STUDIES ON KIDNEY HOMOTRANSPLANT REJECTION USING A CROSS-CIRCULATION TECHNIQUE

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The accelerated destruction of second-set homografts^{1, 2, 3, 4} is best explained on an immunological basis, and the solution to the homograft problem appears to depend on an understanding of the immunological reactions involved. The bulk of recent evidence indicates that both circulating blood cells and plasma antibodies are concerned in homotransplant destruction. Algire *et al.*⁵ placed homografts inside chambers, the plastic walls of which had minute pores large enough to admit plasma proteins but too small to permit the host's blood cells to enter. Under these circumstances homografts persisted indefinitely. The grafts were destroyed, however, if the pores were large enough to allow the host's blood cells to enter the chamber and come in contact with the graft. Good and Varco,⁶ in skin-grafting experiments in agammaglobulinemic patients, have shown that such cases accept homografts permanently, despite evidence that the hosts can develop bacterial-type hypersensitivity. The latter findings seem to indicate that a plasma antibody also is necessary for homograft rejection.

Another interesting recent development concerns the production of "actively acquired tolerance," in which animals injected *in utero* or in the immediate postnatal period with cells from an animal of another strain will later permanently accept grafts from any member of that strain.⁷ Hardin and Werder⁸ achieved a high percentage of successful permanent skin homograft survivals in mice that had been injected previously with donor skin. This phenomenon finds an immunological precedent in Felton's⁹ immunoparalysis, although the exact parallelism is not yet defined. The enhancement of tumor transplants by previous injections of lyophilized tumor extract¹⁰ seems to be based on similar mechanisms.

Our experiments, using a cross-circulation technique that allows a thorough mixing of the blood streams between a pair of animals,¹¹ were designed to clarify certain aspects of the development of homograft immunity. Simonsen¹² carried out repeated injections of blood from a proposed donor to the future host and stated that "immunization" developed in 2 instances so that a subsequent kidney transplant behaved like a second-set graft. It was our intention to see if cross circulation would likewise result in immunization of the animals involved, or if the prolonged periods of circulatory admixing might instead result in an immunoparalysis of antibody-forming tissues to the circulating antigen of the other animal, thus permitting successful homografting between the animals at a later date.

Cross circulation was performed for periods ranging from 5 minutes to 3 days between pairs of mongrel dogs, at flow rates of from 250 to 800 cc. per minute, as determined with a rotameter. The animals were partially heparinized, and

an artery of each was connected to a vein of the other through polyethylene tubes. From 1 week to 120 days after cross circulation a kidney transplantation was performed from one animal of a pair to the other, and the function of the transplant was followed through daily collections of urine. Eleven of the 15 transplants functioned for less than 1 day, and the series was in every way comparable to a previous series of second-set kidney homotransplants done in our laboratory.¹ The average period of function of the transplants done between the previously cross-circulated animals was 1.8 days, as compared to 1.6 days in a series of secondary renal transplants,¹ and 5.9 days in our series¹⁶ of primary transplants. It is evident that cross circulation is a very effective means of producing immunity in 1 animal to the tissues of another. Furthermore, the immunity was shown to last at least 120 days.

In other experiments we found that one transfusion of 500 cc. of blood from a proposed donor to a future host did not produce immunity, although cross circulation for as little as 5 minutes at 500 cc. minute did. This suggests that the amount of blood that must be exchanged in order to achieve immunity lies somewhere between 500 and 2500 cc. The longer periods of cross circulation (over 24 hours) seemed to set the stage for more rapid and intense rejection of the subsequent renal homograft.

It is probable that a state of immunity is not reached until several days after cross circulation. Transplants done 7 days after cross circulation behaved like secondary transplants, while those done on the day of cross circulation behaved like primaries.

It is of note that in 2 instances cross circulation failed to produce immunity, as shown by the fact that a subsequent renal transplant behaved like a primary. This same phenomenon was also noted in 2 of 15 secondary transplants performed by us after a previous primary transplant had been rejected.

A series of primary renal homotransplants was carried out in dogs whose blood types had been determined prior to transplantation. It was found that host anti-red-cell antibody formation did not bear a direct relationship to transplant rejection. The transplant with the longest period of function and survival occurred in an animal forming a high titer of antibodies to the red cells of the donor. It therefore appears likely that either the white cells or the plasma proteins, or both, are the effective immunizing agents, or alternatively that the red cells possess 2 different types of antigens. There is some evidence suggesting a possible relationship between blood groups and kidney¹³ homotransplants in man. The situation in the dog, who does not have naturally occurring blood group antibodies, and that in man, who does, may not be strictly comparable. In this connection, it is of interest that Medawar,¹⁴ while able to produce it with lymphocytes, failed to produce immunization with red cells in rabbits. He also noted that the intradermal injection of lymphocytes was much more effective than through the intravenous route, and he attributed this result to the fact that an intradermal injection is virtually a lymphatic injection. Our data indicate that intravenous immunization with a sufficient antigenic dosage can be extremely effective. We have recently found that skin transplants between rabbits previously cross-circulated for 1 hour

behaved like second-set grafts, thus confirming the immunizing ability of short periods of cross circulation.

Having thus demonstrated the ability of cross circulation to immunize the host to subsequent transplantation, we were interested in learning whether antibodies to the donor's cells present in the host would damage the donor's tissues when the 2 animals were linked in cross circulation. Therefore a kidney was transplanted from 1 animal to another and was observed to exhibit the usual primary homotransplant rejection, with at least 5 days of urine output over 100 cc. and typical gross and microscopic features. Several varieties of cross-circulation routines were used:¹⁵ some pairs of animals were joined for 15 minutes on each of 7 days after homotransplant removal, while other pairs were joined for from 3 to 24 hours on a single or on several occasions from 1 to 2 weeks after transplant removal. Biopsies of the remaining donor kidney, lung, and spleen were performed at 6 and 10 days after the last cross circulation. In 9 such experiments no significant gross or histological changes were observed in donor or recipient organs, and the remaining donor kidney appeared in all ways normal. No delayed destruction occurred in the remaining donor kidney, as was proved by the continued vigor of all animals for many months.

An experiment was designed to permit the antibodies present in the host to perfuse the donor kidney directly without having first to pass through the tissues of the host (as in the usual cross-circulation techniques), where donor antigen might neutralize the antibody before it reached and was fixed to the kidney. In 1 instance the carotid artery of the host was joined to the renal artery of the donor's remaining kidney, the carotid artery of the donor being at the same time joined to the jugular vein of the host. Despite one-half hour of host blood perfusion into the remaining donor kidney, no histological changes were observed therein during the postcross-circulation period. It must be emphasized, however, that after a 5-minute period the blood of the 2 animals was thoroughly mixed, and the donor kidney was thus exposed directly to the "unneutralized" antibodies for only a very short time.

There appear to be 2 possible explanations for the lack of changes in the remaining donor kidney in these experiments: (1) absorption of antibody by donor tissues or circulating antigens, resulting in a dilution effect with insufficient antibody reaching the remaining kidney to result in changes; and (2) the presence of a circulating protective substance in the donor that stabilizes his own tissues and is individual-specific. It is hard to ascertain which of these possibilities is functional in these experiments.

The next logical experiment to present itself as a result of these studies was one in which the transplantation and cross circulation were performed simultaneously. A technique of chronic cross circulation was used; here the animals were in continuous circulatory junction for 2 to 4 days. Using 2 dogs that, 2 weeks previously, had been cross circulated for 3 days (and should, therefore, have been immunized), cross circulation and simultaneous transplantation was performed. The kidney transplant secreted over 200 cc. of clear urine for 3 days; at that time, a biopsy of the transplant was performed and the animals were separated from cross circulation. The biopsy was completely normal and,

after the cross circulation was discontinued, the transplant went on to rapid necrosis with gross and microscopic features of a secondary homotransplant. In 3 other experiments, transplantation and simultaneous cross circulation were performed; biopsies of the transplants were normal at 2, 3, and 4 days. In a previous series of transplantations with daily serial biopsies we have shown that mononuclear cell infiltration is almost always present by this time in primary homotransplants.¹⁶ These experiments again demonstrated the fact that there is protection of the transplant as long as the circulatory systems of the animals are in continuity, even if they have been previously "immunized" and an explosive transplant reaction might be expected. Kamrin and Kamrin¹⁷ have shown that kidney slices exchanged between parabiotic rats survive as permanent homografts as long as the rats remain in parabiosis. This would seem to be another example of homograft protection by the exchange of body fluids.

The present experiments demonstrate that this protective effect, as well as immunization itself, can be brought about through blood stream intermixing alone, and does not depend on tissue contact or lymphatic drainage. Our present data do not permit us to adduce whether neutralization of host antibodies by tissue or blood-donor antigens is responsible for the protective effect of cross circulation or whether some individual specific protective substance is created by the donor for this task.

References

1. MEDAWAR, P. B. 1944. The behaviour and fate of skin autografts and skin homografts in the rabbits. *J. Anat.* **78**: 176-199.
2. DEMPSTER, W. J. 1953. Kidney homotransplantation. *Brit. J. Surg.* **40**: 447-465.
3. SIMONSEN, M., J. BUEMANN, A. GAMMELTOFT, F. JENSEN & K. JORGENSEN. 1953. Biological incompatibility in kidney transplantation in dogs. 1. Experimental and morphological investigations. *Acta Pathol. Microbiol. Scand.* **32**: 1-35.
4. EGDAHL, R. H. & D. M. HUME. 1955. Secondary kidney homotransplantation. *Surg. Forum Proc.* **6**: 423-427.
5. ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1954. The growth of cells in vivo in diffusion chambers, survival of homografts in immunized mice. *J. Natl. Cancer Inst.* **15**: 493-502.
6. GOOD, R. A. & R. L. VARCO. 1955. Successful homograft of skin in a child with agammaglobulinemia. *J. Am. Med. Assoc.* **167**: 713-716.
7. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1953. Actively acquired tolerance of foreign cells. *Nature.* **172**: 603-606.
8. HARDIN, C. A. & A. A. WERDER. 1955. A one year study of surviving homografted mouse skin. *Plast. & Reconstr. Surg.* **16**: 107-113.
9. FELTON, L. D. 1949. The significance of antigen in animal tissues. *J. Immunol.* **61**: 107-117.
10. KALISS, N. 1955. Induced alteration of the normal host-graft relationships in homotransplantation of mouse tumors. *Ann. N. Y. Acad. Sci.* **69**(3): 385-391.
11. EGDAHL, R. H. 1955. Physiological basis of uncontrolled cross circulation in dogs. *Am. J. Physiol.* **182**: 454-459.
12. SIMONSEN, M. 1955. The acquired immunity concept in kidney transplantation. *Ann. N. Y. Acad. Sci.* **69**(3): 448-453.
13. HUME, D. M., J. P. MERRILL, B. F. MILLER & G. W. THORN. 1955. Experiences with renal homotransplantation in the human: report of nine cases. *J. Clin. Invest.* **34**: 327-382.
14. MEDAWAR, P. B. 1947. Immunity to homologous grafted skin. 2. The relationship between the antigens of blood and skin. *Brit. J. Exptl. Pathol.* **27**: 15-24.
15. EGDAHL, R. H. & D. M. HUME. 1956. Immunological studies in renal homotransplantation. *Surg. Gynecol. Obstet.* **102**: 450-463.

16. HUME, D. M. & R. H. EGDAHL. 1955. Progressive destruction of renal homografts isolated from the regional lymphatics of the host. *Surgery*. **38**: 194-214.
17. KAMRIN, B. B. & R. P. KAMRIN. 1955. Auto and homotransplantation of kidney slices in single and parabiotic rats. *Anat. Record*. **122**: 223-241.

Discussion of the Paper

BENJAMIN B. KAMRIN* (*State University of New York College of Medicine, New York, N. Y.*): The work reported by Egdahl and Hume demonstrating the eventual inability of massive cross-circulation techniques to protect and sustain whole-kidney homografts is most interesting. In many instances, the results of their investigations on nonlittermate dogs closely parallel the results obtained in our study of reciprocal homografting of kidney tissue to separated parabiont albino rats.

The various experiments reported below were all done on littermate albino rats in parabiotic union or after separation from such union. Where such animals are placed in successful parabiosis (criteria: complete obliteration of suture line by the 28th day of union, equal growth of united parabionts, and equal growth of hair with no areas of alopecia¹) it is assumed that the resulting kidney-tissue and skin compatibility is due to immunological paralysis or long-acting antibody-antigen neutralization.

Unlike Egdahl and Hume's cross-circulation methods, in which about 600 cc. of blood was exchanged per minute between their experimental dogs, the 100-gram parabiosed rat pairs exchange approximately 150 cc. of blood every 24 hours.² After attaining a total weight of 250 grams, the parabionts may exchange as much as 600 cc. every 24 hours. In addition, the dogs were immunized by the intermingling of the blood (600 to 800 cc. minute) by cross circulation for periods as short as 5 minutes, so that subsequent renal transplants between these animals acted as second-set grafts do. Significantly, this phenomenon was not observed in separated albino rat parabionts that had formerly been in successful parabiosis, that is, initial kidney-tissue homografting at the time of separation and challenged 30 days later with a second set of reciprocally exchanged homografts. These grafts were not rejected for the duration of the experiment. This experiment and others are described below.

Successful homografting of kidney tissue. After newly weaned littermate albino rats have been placed in parabiosis and have achieved a compatible successful status (this occurs in 25 per cent of all the surgically united pairs after 28 days of union), the adjacent kidneys are exposed, one third of the kidney excised and reciprocally transplanted. The homografted kidneys are replaced in the abdominal cavity and the wounds sutured. When such grafted kidneys are removed after remaining within the parabiosed animals for periods up to 92 days, it is found that 50 per cent of the grafts have formed viable and functional unions with the host.³ FIGURE 1 illustrates such a section from a grafted kidney 56 days after homotransplantation.

Separation of successful parabiosed pairs and subsequent transplantation of kidney tissue. Reciprocal homografting of kidney tissue performed after separation of successful parabionts results in the retention of a phantomlike

* ROBERT P. KAMRIN, University of Pennsylvania School of Medicine, Philadelphia, Pa., collaborated in the initiation of these investigations.

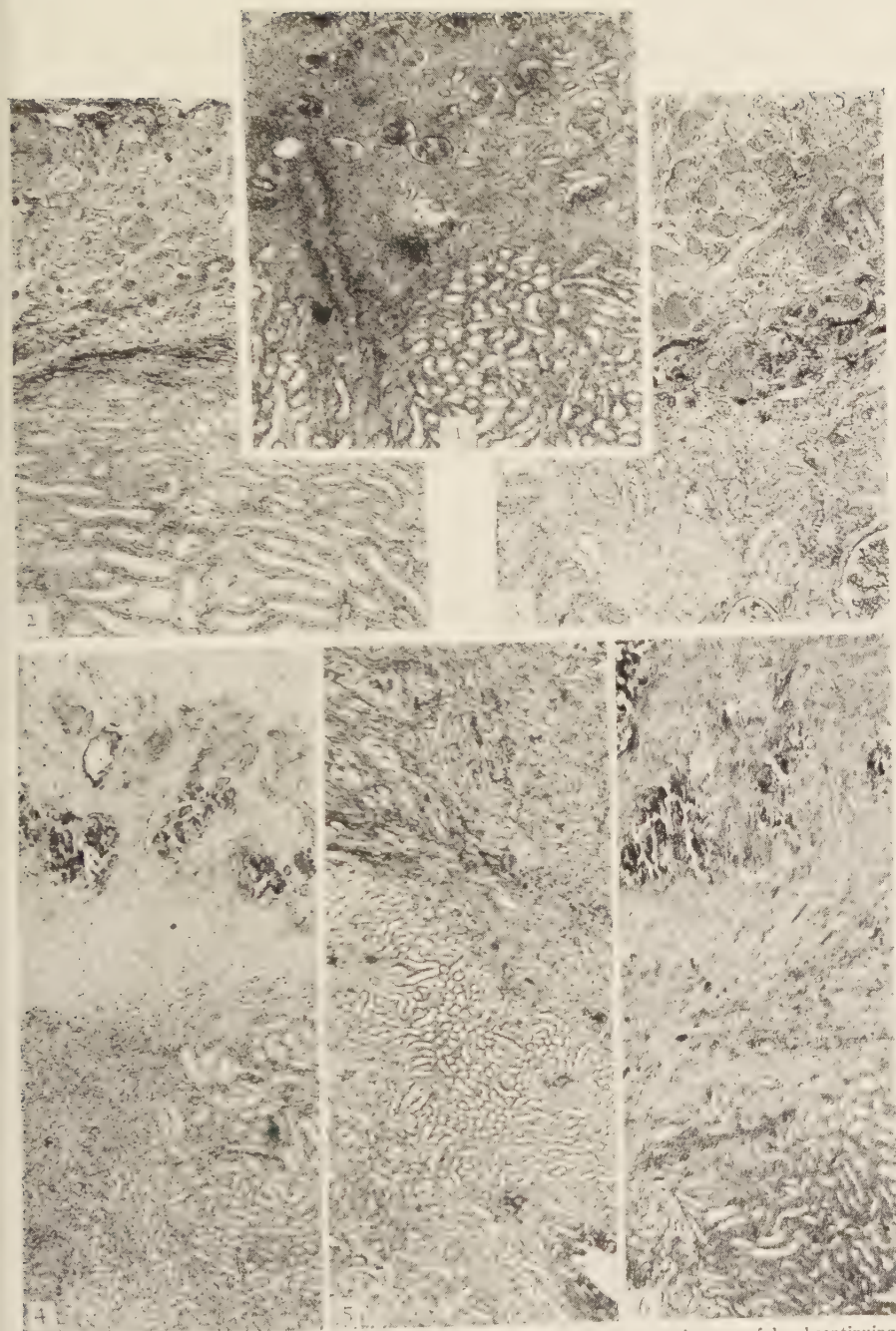


FIGURE 1. Reciprocal homograft of kidney tissue exchanged between albino rats in successful and continuing parabiosis for 56 days (graft on top, host below). $\times 70$.
 FIGURE 2. First-set reciprocal homografting after separation of successful parabionts, *in situ* 71 days. $\times 70$.
 FIGURE 3. Second-set reciprocal homografting performed 30 days after first set, *in situ* 41 days. $\times 70$.
 FIGURE 4. Donor homograft (top) transplanted 30 days previously following 5 intraperitoneal injections of donor kidney homogenate. $\times 55$.
 FIGURE 5. Donor homograft (top) transplanted 30 days previously following 7 intraperitoneal injections of donor kidney homogenate. $\times 55$.
 FIGURE 6. Donor homograft (top) transplanted 30 days previously following 10 intraperitoneal injections of donor kidney homogenate. $\times 55$.

graft that contains nonviable parenchymal structures and a well-vascularized stroma. FIGURE 2 shows a typical example 71 days postoperatively. If this first set of homografts is followed by a second set of homografts reciprocally exchanged between the unoperated pair of kidneys about 30 days later (FIGURE 3), a similar graft appearance is obtained. The host tissue near the graft bed shows a greater degree of reactivity manifested by lymphocyte infiltration and closure of the lumen of adjacent proximal tubules.⁴

Simultaneous parabiosis and homografting of reciprocal kidney tissue. The results achieved in this experiment on 37 pairs of albino rats strongly suggest that either immunological paralysis of the host occurs during the first week of homografting or that the parabiotic union neutralizes the antigenicity of the grafts. Permitting the animals to remain in parabiosis for 28 days after homografting also permits retention of viable appearing homografts. Separation of the parabionts apparently abrogates the neutralization mechanism, and slow destruction of the parenchyma then occurs. Grafted kidneys in animals that underwent 14 to 21 days of parabiosis and a subsequent 14 to 21 days of separation, appeared necrotic and in some cases were rejected entirely. This procedure, which combined features of the above 2 cited experiments, suggests that the act of successful parabiosis is conducive to submerging any antigenic action of the graft, but that this property is slowly lost when the animals return to their individual status.

Kidney homogenate as an enhancing factor in homografting. Fourteen pairs of animals in successful parabiosis were separated, and a nonlittermate donor allocated to each pair. The right kidney of each donor was removed as needed and homogenized with 7.5 cc. of physiological saline. The formerly parabiosed pairs were divided into 3 groups. The right animal of Group I received 5 intraperitoneal injections of 0.5 cc. of homogenate on alternate days, followed by kidney-tissue homografting of the donor-remaining kidney to both the right and left (control) animals. The kidney sites were prepared by excising a piece the size of the homograft and planting the homograft. Group II received 7 homogenate injections, while Group III received 10 homogenate injections, with the same subsequent procedure of homografting. None of the homografts placed in the homogenate-treated animals were found to be viable after 30 days *in situ*. FIGURE 4 shows the results of homografting after 5 homogenate injections. The graft parenchymal structures are encapsulated in necrotic areas, although some individual phantoms of glomeruli and tubuli are found. The graft stroma is highly vascularized and contains numerous lymphocytes distributed throughout the tissue. After receiving 7 homogenate injections (FIGURE 5), the graft preserves more of the phantom appearance against a background of connective tissue fibers. The injection of 10 homogenate solutions (FIGURE 6) appears to cause a hypersensitivity of the host. In three animals, the graft was encysted by a thick connective-tissue capsule; in 2 animals, the representative section shown here demonstrated necrosis and connective-tissue infiltration. In all cases, the host tissue adjacent to the graft bed showed little reaction.^{5,6}

It should be noted that the control homografts from a nonlittermate donor showed the same reaction as that seen in reciprocally exchanged homografts.

Summary. The experimental findings clearly demonstrate that albino rats in successful and continuing parabiosis elaborate sufficient enhancing substance for the retention and survival of the homograft. This blocking of the antigenicity of the graft is slowly eliminated if the animals are separated after short periods of parabiosis. Sufficient quantity or effectiveness of the substance remains, however, to prevent necrosis and sloughing of the homograft. Earlier experiments demonstrated that kidney-tissue homografts exchanged between littermates that *had never been* in parabiosis were sloughed off between the 7th and 11th postoperative day. It is assumed that the mechanism involved in these experiments is of an immunological nature.

References

1. KAMRIN, B. B. 1954. J. Dental Research. **33**: 824.
2. HUFF, R. L., R. TRAUTMAN, D. C. VAN DYKE. 1950. Am. J. Physiol. **161**: 223.
3. KAMRIN, B. B. & R. P. KAMRIN. 1955. Anat. Record. **122**: 223.
4. KAMRIN, B. B. 1956. Anat. Record. **124**: 541.
5. KAMRIN, B. B. 1956. Anat. Record. **124**: 315.
6. KAMRIN, B. B. 1956. J. Urol. **76**: 142.

THE RELATION OF HAIR CYCLES TO THE SURVIVAL TIME OF SUPRAPANNICULAR AND SUBPANNICULAR SKIN HOMOGRAFTS IN RATS*

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The cyclic growth of hair in young rats was demonstrated by Butcher (1934), who reported that the growth of hair follicles in the first cycle extends from birth to the 16th or 17th postnatal day and becomes inactive by the 22nd day. This inactivity, described as a quiescent or rest period, persists until the 32nd day, when active growth and lengthening of the hair follicles is resumed.

We have attempted to correlate the cycles of hair growth in rats with the duration of skin homografts, acting on the hypothesis that if appreciable differences in survival time could be related to phases of the hair cycle, such variations would be of interest to workers in the field of tissue transplantation.

Two types of skin grafts were used in homotransplantation. In one, the skin graft was removed from above the panniculus carnosus (suprapannicular grafts); in the other, the skin, together with the panniculus (subpanhicular grafts), was removed. The relative survival time of each of these homografts was studied in this series of experiments.

Methods and Techniques

Wistar, Sherman, and Long-Evans strains of female rats were grouped according to age: 22, 32, and 42 days respectively. These different age-group rats were selected because, according to Butcher (1934), at 22 days old the hair follicles are at a resting stage, at 32 days old the hair follicles have resumed growth, and at 42 days old the hair follicles have attained their maximum depth. Suprapannicular and subpanhicular homografts were performed on animals of the same age group.

The animals were anesthetized with intraperitoneal injections of Nembutal, and the hair on the right side of the thorax was clipped and shaved. In each case hair clipping was done immediately before homografting in order to minimize any possible effect on the healing rate, as hair clipping appears to exert a stimulus on wound healing (Sandblom and Muren, 1954). If clipping of the hair were done at various time intervals before grafting, the stimulus to wound healing could be considered a possible source of error. The shaved skin was washed and cleansed with 70 per cent alcohol and ether. The dimensions of the grafts, all removed from the right side, were 12.5×10 sq. mm.

Suprapannicular grafts were removed in a group of 10 rats by incising through the dermis and carefully peeling the graft from the panniculus carnosus by sharp dissection. Subpanhicular homografts were removed in another group of 10 rats. In these the incision was extended through the pannicular layer, and the edge of the surrounding skin was raised with forceps to facilitate the

* The work described in this paper was supported by grants from the Atomic Energy Commission, Washington, D. C., and the Milbank Memorial Fund, New York, N. Y.

insertion of straight, sharp scissors into the line of incision. The connections of the underlying fascia of the body musculature to the panniculus were severed; the grafts were then removed from the bed with little attendant bleeding.

All suprapannicular and subpannicular homografts were placed into suprapannicular and subpannicular prepared beds respectively, and anchored with interrupted silk sutures. Sterile mineral oil was then applied to prevent drying of the graft, and a small square of nylon cloth was placed to prevent adhesion of the gauze dressing, which was maintained by a strip of sterile gauze around the trunk. Adhesive tape was wrapped around the thorax twice, thus ensuring that sufficient pressure was exerted on the grafts without hindering respiration or free movement. This was covered with a belt fashioned from flexible zinc strips, secured with 2 turns of adhesive tape, to prevent the animal from chewing and tearing the dressing.

Revascularization of the grafts was observed daily through a film of mineral oil under a Bausch and Lomb stereoscopic microscope, following the technique of Taylor and Lehrfeld (1953). The time of skin rejection was determined when hemal stasis, followed by vascular disruption, was observed in the homograft vessels. All the homografts became revascularized, and no infection occurred.

Comparative Surgical Time of Subpannicular and Suprapannicular Homografts

Subpannicular homografts. Upon removal of the subpannicular graft from the donor bed, the surface of the bed became wider, due to muscular contraction of the surrounding panniculus carnosus. The excision produced little bleeding. The excised graft was diminished in size, due to contraction of the underlying pannicular layer and of the elastic tissue in the dermis. Stereomicroscopic observations of the bed revealed a thin layer of fascia and thoracic musculature and, near the surface, intact blood vessels that appeared diminished in caliber. Blood vessels could be seen dividing into plexuses and becoming progressively smaller in diameter toward the epidermis.

On the day following implantation, the graft appeared paler and more pliable than the neighboring recipient skin. Stereomicroscopic examination revealed signs of early distension in the extensive network of blood vessels. No blood flow was observed.

Two-day-old homografts appeared pinkish and pliable, and epithelial growth could be seen at the line of union. Vessel distension continued, but no flow in the vascular network could be observed.

The line of union between graft and host was nearly healed on the third day after implantation, and the graft appeared healthy. There was a marked return of the vessels to normal caliber at this stage, and normal flow was observed in most vessels.

The line of union between graft and host was epithelized and completely healed in the 4-day-old homografts. Stereomicroscopic examination showed a return of all blood vessels to normal caliber and circulation.

From the fourth day until the first signs of rejection appeared there was little change in the appearance of the graft. The earliest evidence of the onset of

rejection was the increased distension of all blood vessels, followed by sluggish circulation with clumped elements. A few thrombosed areas appeared shortly thereafter, particularly in the small capillary loops, and then spread rapidly to the larger vessels. Hemal stasis followed and vascular disruption occurred, usually on the following day.

After the onset of the homograft-rejection period, the graft exhibited gross progressive changes in color, turning various shades of red, yellow, or green; on palpation, the graft had a rigid feel. Occasional homografts were crusted and escharified.

A summary of subpannicular homograft observations is shown in TABLE 1. The mean survival time of 13.0 days for subpannicular homografts in the 22-day-old rats exceeded that of 8.1 days in adult rats (Taylor and Lehrfeld, 1953). The shortest survival time (in 3 rats) was 9 days; and the longest (in 1 rat) was 21 days. The earliest active hemal flow appeared in all the 22-day-old rats on the third postoperative day, whereas in Taylor's and Lehrfeld's rats it occurred on the fourth day.

Suprapannicular homografts. Little if any increase in the surface area of the graft bed was observed after removal of the homograft. The graft failed to contract upon itself, although such contractions had occurred in the subpannicular homografts. When examined under the stereomicroscope, the under-surface (the dermis of the graft) showed a thin layer of loose areolar connective tissue containing a considerable number of fat cells and hair follicles. Numerous vessels in the graft were of smaller caliber than those in the subpannicular homograft. Secondary blood vessels were observed coursing through the panniculus carnosus layer in the host bed. Larger vessels were visible just beneath this layer.

Hemal stasis and the onset of the homograft-rejection period did not appear

TABLE 1
SUBPANNICULAR HOMOGRAFTS

22-day-old rats		32-day-old rats		42-day-old rats	
No. of rats	Day blood flow ceases	No. of rats	Day blood flow ceases	No. of rats	Day blood flow ceases
3	9	2	6	1	6
5	10	2	7	5	7
4	11	6	8	5	8
3	12	4	9	3	9
4	13	2	10	2	10
1	14	1	11		
4	15	1	14		
3	17	1	15		
1	18	1	16		
4	19	1	17		
1	21	1	19		
33	429	22	222	16	128
Mean average = 13.0		Mean average = 10.1		Mean average = 8.0	

TABLE 2
SUPRAPANNICULAR HOMOGRAFTS

22-day-old rats		32-day-old-rats		42-day-old-rats	
No. of rats	Day blood flow ceases	No. of rats	Day blood flow ceases	No. of rats	Day blood flow ceases
1	8	3	7	1	6
5	9	5	8	2	7
1	10	3	9	4	8
3	11	3	10	1	9
3	12	1	11	4	10
3	13	2	12	1	11
1	14	1	14		
2	15	2	16		
4	16				
2	18				
1	19				
3	20				
2	21				
1	22				
1	23				
33	481	20	205	13	112
Mean average = 14.5		Mean average = 10.2		Mean average = 8.6	

before 8 days in 1 suprapannicular graft and not later than 23 days in another (TABLE 2). The mean time at which hemal stasis occurred was 14.5 days, exceeding that of 8.1 days reported by Taylor and Lehrfeld (1953) in their series of suprapannicular homografts. The mean survival time of the suprapannicular homografts (14.5 days) also exceeded that of the subpannicular homografts (13.0 days) by 1.5 days.

Postoperative observations of the course of events in subpannicular and suprapannicular grafts were quite similar. The vascular network showed active hemal flow by the third day.

Discussion and Conclusions

In these experiments, both subpannicular and suprapannicular skin homografts survived for a longer period of time during the quiescent stage of the hair cycle than in the growth period.

The time of onset of the homograft-rejection period in 22-day-old rats occurred later than in the series of adult rats (8.1 days) reported by Taylor and Lehrfeld (1953). TABLES 1 and 2 showed a slight difference in the mean survival time of subpannicular and suprapannicular homografts in the same age group. The onset of active hemal flow in homografts occurred one day earlier in the animals during the quiescent period than after active hair growth was resumed. Other characteristics of the homograft-rejection period closely resembled the description given by Taylor and Lehrfeld (1953) in adult rats.

Comparative survival time of homografts in 22-, 32-, and 42-day-old rats. Except for the length of survival time, the gross and stereomicroscopic observations revealed that there was very little divergence in appearance, behavior,

and fate of either subpannicular or suprapannicular homografts among the rats of these 3 age groups. In the 42-day-old rat homograft active hemal flow occurred somewhat later, usually on the fourth day, than in homografts in the animals of the other age groups.

It is of interest to note that both subpannicular and suprapannicular homografts persisted for longer periods in the 22-day-old rats. The variation in the onset of the homograft-rejection period is from 9 to 21 days in the subpannicular grafts for the 22-day-old rats, with a mean survival time of 13 days, whereas the rejection period is from 8 to 23 days in the suprapannicular grafts, with a mean survival time of 14.5 days (TABLES 1 and 2). These results are similar to the survival time of skin homografts in the 22-day-old rats (TABLE 2).

Hemal stasis occurs earlier in the 32-day-old rats, between the 7th and 16th day in the suprapannicular homografts, and between the 6th and 19th day in the subpannicular homografts, with a mean survival time of 10.2 and 10.1 days, respectively. This seems to indicate a more rapid rejection of homografts in 32-day-old rats than in 22-day-old rats.

The rejection time decreased as the age of the rats increased (TABLES 1 and 2). Data illustrated in TABLES 1 and 2 show that the mean survival time is 8.6 days and 8.0 days in suprapannicular and subpannicular homografts, respectively, in 42-day-old rats. This coincides with the 8.1 days previously reported by Taylor and Lehrfeld (1953).

Suprapannicular homografts appeared to survive somewhat longer than the subpannicular grafts of the corresponding age group. The difference, however, does not seem significant.

One or more common factors associated with the hair-growth cycle are determining factors in the survival time of homografts. All homografts, either subpannicular or suprapannicular, show the longest survival time when the hair follicles are at a resting stage, as in the 22-day-old rats. When the quiescent period terminated, as in the 32-day-old rats, the survival time of both types of homografts shortened. Furthermore, when the hair follicles reach their maximum depth, as in the 42-day-old rats, the rejection of both types of implantation occurs more rapidly than in the other age groups. It is of interest to note (TABLES 1 and 2) the wide variations in survival time of homografts in 22-day-old rats. In the 32- and 42-day-old animals, the variations tend to decrease; in the adult rats, survival time tends to become constant. The increasing age in the animals, however, might also be a determining factor in the survival time of homografts. Further experiments are being undertaken to determine whether the hair-growth cycle or the age of the animals is the principal cause.

Acknowledgments

Thanks are expressed to Harry H. Shapiro for editorial assistance and to Lawrence Rosenstock for technical assistance.

References

- BUTCHER, E. O. 1934. The hair cycles in the albino rats. *Anat. Record*, **61**: 5-13.
SANDBLUM, P. H. & A. MURIN. 1954. Differences between the rate of healing of wounds inflicted with short time interval. *Ann. Surg.* **140**: 449.

TAYLOR, A. C. & J. W. LEHRFELD. 1953. Determination of survival time of skin homografts in rat by observation of vascular changes in graft. *Plast. Reconstr. Surg.* **12**: 6.

Discussion of the Paper

PETER RANDALL (*Harrison Department for Surgical Research, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*): I congratulate Donald L. Ballantyne, Jr., and John Marquis Converse on their work and presentation. The end point that Ballantyne and Converse have used regarding the possible variations that might be caused by the cyclic changes in the skin is abrupt. Nevertheless the duration of survival has decreased from 14.5 days to 8.6 days, depending on whether the grafting was done within the resting stage or within the actively growing stage of the cycle. The implications of this result are certainly very great.

The work on the effect of skin cycles on the duration of survival of skin homografts in mice that Ira M. Dushoff and I have undertaken is still incomplete, but the results thus far seem to be comparable to those presented in the paper under discussion.

Skin cycles have been described as a regular waxing and waning of the physiological activities of the skin in rhythmical fashion. For example, all the hair in a given area will pass through a growing phase called anagen, a very short regressive phase called catagen, followed by a rather prolonged resting phase of complete inactivity called telogen. These cycles have been well described in the mouse and the rat, but they also occur in all other rodents with the exception of the guinea pig.

First I suggest that, for our purposes, the term "hair cycles" be replaced by the term "skin cycles." The reason is that, even though these cycles are most conspicuously reflected in the growth of hair, they apparently affect practically all the physiological activities of the skin, many of which are as important as, or even more important to, transplantation than the hair alone.

FIGURE 1 is a schematic representation of some of these changes as seen in a given area of skin. All of the hair follicles in one area will undergo these changes simultaneously. The marked development of the hair follicles in the first 8 days of anagen is very striking and has been subdivided into 6 stages by

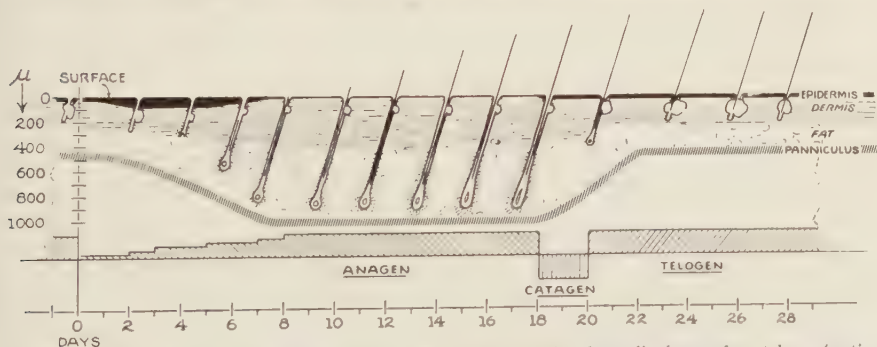


FIGURE 1. Schematic drawing of a skin cycle—mouse or rat—showing the cyclic changes from telogen (resting phase) on the left, through anagen (growing phase), and catagen (regressive phase), to telogen on the right. Hair follicles, sebaceous glands, and capillaries (small dots seen around the growing hair follicles) are shown but not labeled.

Chase *et al.*¹ The hair follicle elongates about 3 times its resting length, extending down to the muscular panniculus carnosus; during this stage of development, considerable mitotic activity is seen in the developing hair follicle. The new hair shaft reaches the surface about the fifth or sixth day in what is called anagen V, and all of the hair growth throughout the entire cycle is confined to the period between this stage and the end of anagen on about the 18th day. During the 2 days of catagen there is an abrupt cessation of this activity with a marked regression and shortening of the hair follicle to its resting length. This shortening causes a further extrusion of the hair shaft above the hair surface.² Telogen in the mouse lasts about 10 days and in the rat about 17 days; during this period of time the hair follicle rests in a vegetative condition.

These marked changes have given rise to the term "hair cycles," and yet the figure illustrates many other changes synchronized with the cycle of the hair. The epidermis, for example, develops considerably in thickness in the early stage of anagen, going from a resting thickness of about 25 μ to about 75 μ with considerable mitotic activity. With the appearance of the hair shaft at the surface, the epidermis thins to about 15 μ , maintaining this thickness until the onset of telogen, when it again resumes the 25- μ thickness.

The dermis is seen to increase about 50 per cent in thickness during anagen, while the adipose layer enlarges 2 to 3 times, with a marked increase in the deposition of fat. The sebaceous glands are interesting in that they are rather quiescent during anagen whereas, during catagen, they develop and enlarge. Most of the output of sebum is seen during telogen.

The blood supply increases in areas of cellular activity so that, during the active growing and producing stage, a very marked increase is seen in the capillary network around the hair follicle, particularly around the lower one third of the follicle. With the onset of catagen, this development regresses. With the increased activity of the sebaceous glands, however, capillary development is still seen in this area. Alkaline phosphatase activity parallels fairly closely these capillary changes.

These changes are rather profound and it would seem that the variations during the skin cycles would affect almost any type of experiment that utilizes the skin of rodents. Experiments in which the period of observation or exposure is short, such as skin homograft experiments, wound-healing experiments, and experiments involving exposure to radiation and short exposure to carcinogens would probably fall in this category.

Second, the experiments of Ballantyne and Converse have been carried out on very young animals. Cannon and Longmire,³ for example, have shown that day-old chicks will tolerate a skin homograft better and longer than older chicks. The decrease in graft-survival time as seen in these experiments may be due to an increase in the age of the animals, and this possibility has not been ruled out. We have heard much about the importance of immunological maturity; indeed, these animals do not reach sexual maturity until 50 to 60 days. Accordingly, it might be worthwhile to carry out a fourth set of grafts during telogen of the second generation (at about 55 days) to see whether their survival time would approximate that of the grafts transplanted at 22 days of age (FIGURE 2).

Third, it has been pointed out that age is not an accurate means of determining the stage of the skin cycle, particularly after the first generation. For example, even during the first generation there is a lag of 4 to 5 days between the onset of anagen in one area of the body and the onset of anagen in another area. With the second generation this lag can increase to as much as 7 days, and it has been shown that within a 2 cm. strip of skin from the dorsum, several stages can be seen at one time.⁴

Perhaps this partially explains the difference between the shortest and the longest survival time in any one group of animals in the experiment just reported.

Accordingly, it would seem desirable to have a method of controlling the skin cycles in a given area and likewise to be able to use mature animals. It has been shown that injury to a hair follicle in the resting stage of telogen will stimulate the onset of the anagen phase in that area. This can, of course, occur from bites or scratches and result in the production of very bizarre patterns. It can also be induced by plucking the hair of a given area either by hand or with several applications of an epilating wax. When this is done in telogen, a new cycle begins almost immediately, and can be recognized by a thickening of the skin in 5 or 6 days and by the beginning of hair growth by the eighth day. In animals with pigmented hair, this hair growth is noted by a marked darkening of the skin and, in albino animals, by a sudden change in the texture of the surface. Telogen is recognized by the thinness and pliability of the skin.

A cycle thus induced is for all intents and purposes a perfectly normal cycle, and the area plucked will continue on its new time schedule. Plucking in catagen produces the same result, while plucking in late anagen differs somewhat in that the growing phase in progress continues to completion, with anagen then usually recommencing without the intervening telogen phase. Plucking in early anagen gives very irregular results, since the only hairs exposed are those from previous generations. The first few days of anagen may be mistaken for telogen grossly but, if the hair is plucked at this time, the thickening usually seen in 5 or 6 days should occur irregularly at an earlier time, and hair growth should be noted before the eighth day. An accurate control of the

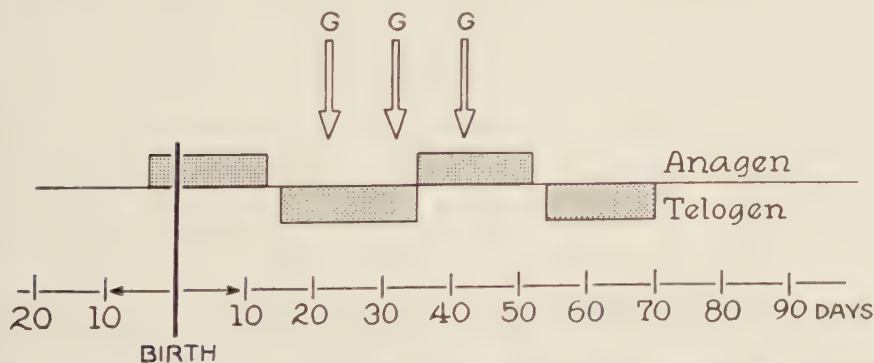


FIGURE 2. Diagram to show the times of grafting (G) as done by Ballantyne and Converse with relationship to the stage of the skin cycle in the rat.

skin cycles can be achieved by waiting until a given area of skin is seen grossly to be in telogen. The hair should then be plucked and the animal observed for the changes expected on the fifth and eighth day. If these changes occur, the stage in the cycle can be determined and future changes predicted.

Again I congratulate Ballantyne and Converse for stimulating interest in the subject under discussion and for illustrating so well the possible effect of skin cycles on the duration of survival of skin homografts. It seems that control of these skin cycles will be important in skin-homograft experiments and also in many other experiments in which the skin of the rodent is used.

References

1. CHASE, H. B., W. MONTAGNA & J. D. MALONE. 1954. Changes in the skin in relation to the hair growth cycle. *Anat. Record*, **116**: 75-81.
2. BUTCHER, E. D. 1934. The hair cycles in the albino rat. *Anat. Record*, **61**: 5-20.
3. CANNON, J. A. & W. P. LONGMIRE. 1952. Studies of successful homografts in the chicken. *Ann. Surg.* **135**: 60-68.
4. WOLBACH, S. B. 1951. The hair cycle of the mouse and its importance in the study of sequences of experimental carcinogenesis. *Ann. N. Y. Acad. Sci.* **53**(3): 517-536

JOHN E. SHANNON, JR. [*Department of Surgery (Plastic), The New York Hospital, Cornell Medical Center, New York, N. Y.*]: I should like to make a brief comment relative to the interesting observations of Ballantyne and Converse on the relation of the hair cycle to the survival of skin homografts. Several years ago an exploratory study was initiated by Conway, Stark, Lazarini, and Sedar, in which adult mouse skin (full thickness) was maintained *in vitro* prior to transplanation. This study indicated that the survival of interstrain homografts could be prolonged after being subjected to short periods of maintenance *in vitro*. In the course of the study it was noted that in some cases (unpublished data) the previously depilated pieces of adult skin grew a fairly luxuriant amount of hair during *in vitro* cultivation. The growth of hair *in vitro*, of course, has been nicely demonstrated with embryonic tissues by Strangeways (1931), Murray (1933), and Hardy (1949). A point of some interest, however, is that when such cultured pieces of skin with actively growing hair were subsequently transplanted as homografts, they appeared to elicit a stronger rejection response and were rapidly destroyed. It is our intention to examine this phenomenon more closely as part of a program designed to determine how fruitful certain applications of tissue-culture techniques might be as an approach to the problem of homotransplantation.

PROLONGED SURVIVAL OF SKIN HOMOGRAFTS IN UREMIC PATIENTS

By Gustave J. Dammin, Nathan P. Couch, and Joseph E. Murray

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In the studies by Hume *et al.*¹ on the functional survival of renal homotransplants in patients with chronic renal insufficiency, it was observed that the period of homotransplant function in 4 of 9 recipients ranged from 5 to 25 weeks. The course of the renal homotransplant in the normal dog contrasts with this wide range and long duration of the period of functional survival of renal homotransplants in the uremic patient. Irrespective of the manner in which the donor and or recipient have been modified, including procedures known to suppress antibody formation, functional survival of the renal homotransplant in the dog seldom exceeds 1 week. Since there has been no uniformly successful experimental counterpart of chronic renal insufficiency in the dog, no information is presently available on the renal homotransplant function in the dog under these circumstances. In man, when the recipient does not have a chronic renal insufficiency, as exemplified by the case of Michon *et al.*,² the renal homotransplant functions well for about 3 weeks and then ceases to function rather abruptly, with the homotransplant showing the same morphologic pattern of rejection seen in the dog. This pattern has been interpreted as a morphologic representation of an antigen-antibody reaction; the immune response of the recipient to the renal homotransplant is believed to be the basis for the rejection. A delayed rejection or prolonged acceptance, therefore, may well represent an impaired immune response. Homotransplantation studies by Dempster³ and Simonsen⁴ have established a close antigenic relationship between the kidney and the skin. Should this relationship apply in man, one would expect the uremic recipient, if the prolonged functional survival is a manifestation of an impaired immune response, likewise to show a prolonged survival of skin homografts. It was on this basis that the study to be described was undertaken.

The first group of patients studied had chronic renal insufficiency with uremia that ranged from 4 months to 6 years in duration (TABLE 1). It may be noted that a variety of chronic lesions is represented, with uremia a common denominator. The upper arm was used as the site for the skin grafts. Skin homografts were accompanied by skin autografts in each case. The grafts were approximately square with an area of 3 to 4 sq. cm. The bed of the recipient site was subcutaneous tissue, and the grafts were full thickness. Biopsies from the homograft and the autograft were obtained simultaneously. Tissues were fixed in buffered 10-per cent formalin and processed through a modified Bouin solution. Hematoxylin and eosin, periodic acid-Schiff, Verhoeff-van Gieson, reticulum, and Feulgen stains were used.

TABLE 2 summarizes the data on the recipient, donor type of homograft, the time interval to biopsy, and the estimate of the degree of homograft survival. "Pure" refers to grafts obtained from normal donors, "cortisone" to grafts from

TABLE 1
UREMIC RECIPIENTS OF SKIN HOMOGRAFTS

Recipient	Group	Uremia	Renal lesion
A.K., 62, M.	B+	6 years	Chronic pyelonephritis
J.F., 27, M.	O+	5 years	Chronic pyelonephritis
J.W., 20, M.	O+	4 months	Hydroneph.; Bl. N. Obstr.
R.M., 26, F.	O+	1 year	Chronic glomerulonephritis
D.B., 33, M.	A+	3 years	Chronic glomerulonephritis
M.S., 53, F.	A+	2 years	Polycystic kidneys
R.H., 23, M.	O+	1 year	Chronic glomerulonephritis

donors receiving cortisone in moderately large doses (for asthma, for example), and "uremic" to grafts from patients in chronic uremia. It will be noted that in each of the recipients at least one of the homografts was graded as having "fair" to "good" survival. The 3 recipients with the longest time interval to the time of biopsy showed homograft survivals rated as "good." Blood-group differences did not appear to influence survival, as shown by recipients M.S., J.F., A.K., and D.B. In recipients M.S. and J.W. good survival of grafts obtained from donors of the opposite sex was noted. The homograft survival in recipient R.H. is graded as "excellent," as might be expected when the donor and recipient are identical twins. At this time, over 15 months after receiving a renal homotransplant from his twin brother, recipient R.H. is well and has only this 1 kidney. His own 2 kidneys, which manifested chronic glomerulonephritis, were removed several months after the renal homotransplantation.⁵

TABLE 2
SURVIVAL OF SKIN HOMOGRAFTS

Recipient	Donor	Homograft	Interval	Survival
M.S., 53, F. A+	R.D., M. A+	Pure	32 days	Good
	R.L., M. O	Cortisone	32 days	Poor
J.F., 27, M. O+	L.L., 57, M. A+	Cortisone	33 days	Good
	J.N., 22, M. O+	Pure	33 days	Poor
	A.K., 62, M. B+	Uremic	33 days	Infection
	R.H., 23, M. O+	Pure	37 days	Excellent
A.K., 62, M. B+	L.L., 57, M. A+	Cortisone	57 days	Fair
	J.N., 22 M. O+	Pure	57 days	Poor
	J.F., 27, M. O+	Uremic	57 days	Fair
	M.S., 59, F. O+	Pure	64 days	Good
J.W., 20, M. O+	M.M., 19, F. O+	Cortisone	115 days	Good
	W.L., 52, M. O+	Pure	115 days	Good
R.M., 26, F. O+				
D.B., 33, M. A+				

The rating of the survival was based on a detailed study of all the structures in the graft and of the adjacent normal skin. Particular attention was paid to the junction between the graft and normal tissue. At the junction in both autografts and homografts, the subepidermal mucopolysaccharide and elastic fibers were absent. In the homografts, portions of these components were invariably present. Therefore, complete rejection of the homograft was not observed. The degree of preservation of the other homograft components (hair follicles, smooth muscle, sweat glands, subepidermal reticulum, collagen, and epidermis), as well as of those just mentioned, was the basis for rating the survival of the homograft. In FIGURES 1 to 6, homografts are compared with the normal skin and a cutaneous scar. Cellular infiltration by lymphocytes, plasma cells, eosinophiles, and mononuclear macrophages was observed in some degree in all of the homografts except in the case of R.H. None of the homografts manifested the type of rejection pattern observed by Converse and Rapaport⁶ and by Ellison *et al.*:⁷ all of the homografts in our study showed more prolonged survival and better preservation. Converse and Rapaport⁶ applied circular full-thickness grafts 11 mm. in diameter to the forearm of normal recipients. Seven days postoperatively they observed edema, a "secondary erythema" surrounding the homograft, and a cherry-red color of the homograft. Swelling appeared, and by the 9th and 10th days the homograft exhibited a

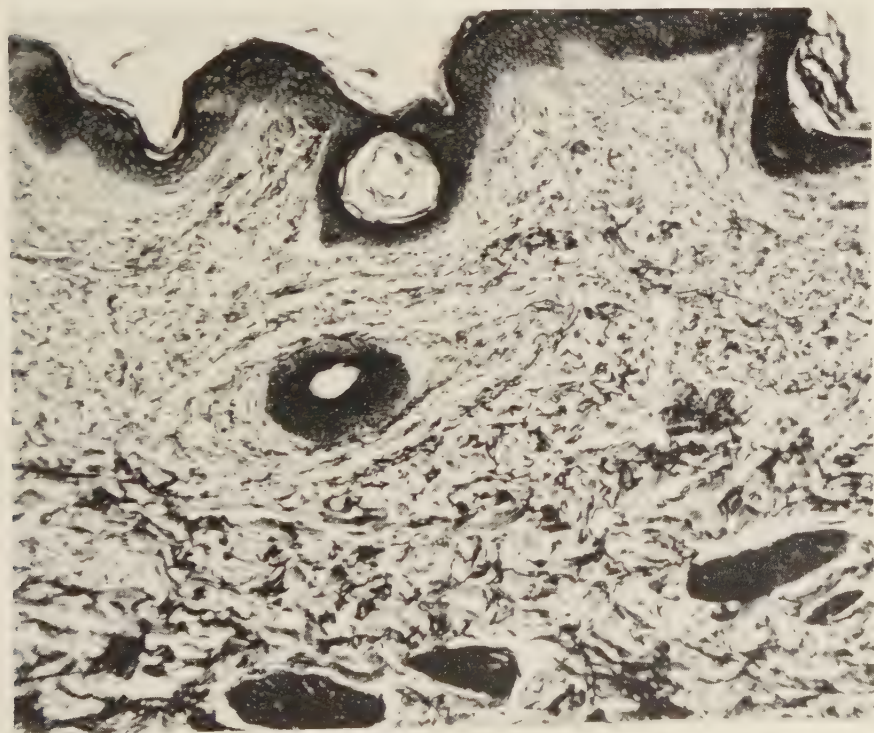


FIGURE 1. Normal skin of recipient D. B. Note the normal elastic fiber pattern, hair follicle, smooth muscle, and epidermis. Verhoeff-van Gieson stain. $\times 115$.

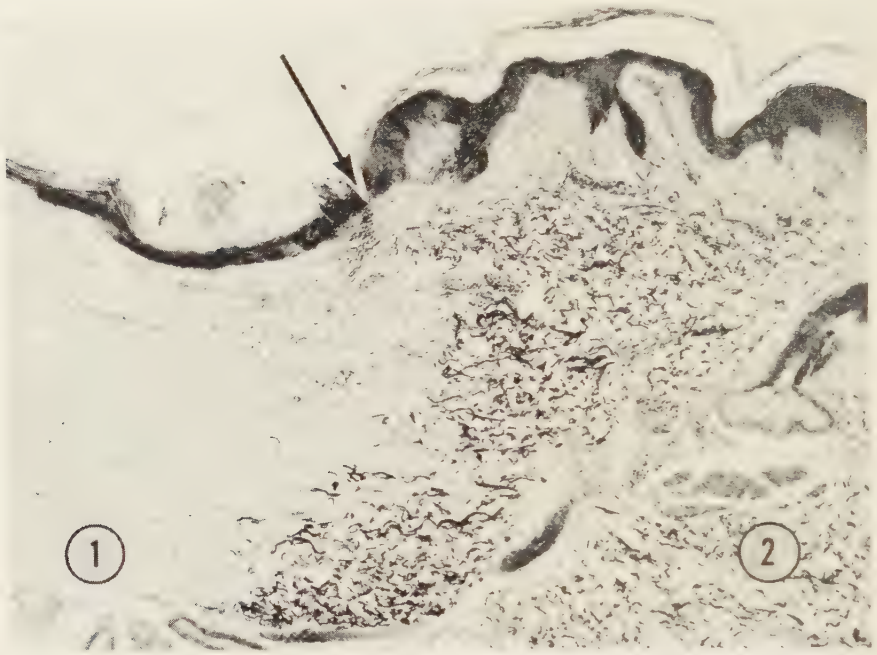


FIGURE 2. Scar of recipient J.F. The arrow denotes the junction of the scar area (1) and the normal area (2) with the rete pegs of the epidermis, the normal elastic fiber pattern, the sweat-gland ducts, the sebaceous gland, and the smooth muscle. Note in scar (1) the thin epidermis without rete pegs, and the absence of elastic fibers and accessory structures. Verhoeff-van Gieson stain. $\times 100$

"pneumatic" appearance. Describing the termination of the experiment, Converse and Rapaport said that "Graft desiccation and escharification followed, leaving a dry and opaque surface on the twelfth or thirteenth day. The graft, a brownish eschar by the fifteenth day, became sloughed by the twentieth day, leaving a dermal pad in the host bed." Ellison *et al.*⁷ studied homograft and autograft survival in burned patients who were receiving adrenocorticotrophic hormone (ACTH) and/or cortisone. These drugs, in the dosage used, were believed not to influence the survival of the homograft. Degeneration in the homografts was observed at 3 weeks, and rejection was complete at 4 weeks.

Chromocenter (sex chromatin) counts were made on the homografts of recipients M.S. and J.W. because the donors were of the opposite sex.⁸ These counts suggested but did not establish the persistence of the homograft epidermis. The pattern of dermal elements in the homograft and the presence of the donor-recipient junction were conclusive evidence of the persistence of the dermis. The epidermal-dermal relationship of the type observed in cutaneous scars (FIGURE 2) was not in evidence in any of the homografts.

In a search of further evidence that this prolonged survival might represent an impaired immune response, the following studies were carried out. Ten adult patients ranging from the fourth to the seventh decade and suffering

from chronic uremia were selected for these studies. Isoagglutinin titers were determined by the Blood Grouping Laboratory of Boston, Inc., Boston, Mass.; all 10 patients were found to have normal isoagglutinin titers. Three of these patients were given an injection of "B" blood group-specific substance. Two of the 3 showed responses comparable to those observed in normal individuals, and the third showed a rise from 1:8 to 1:32. Seven of the patients were given Schick tests. Of these, 6 gave negative reactions. Tuberculin tests were positive in 2 of these 7 patients. Their previous reactivity was not known, and therefore it was not possible to determine the duration of unresponsiveness to tuberculin in those whose tests were negative. The serum of 12 patients in chronic uremia was examined by paper electrophoresis for protein patterns. No depression considered to be significant was observed in the antibody-containing fractions. Gamma globulin was determined quantitatively by the Oudin agar-diffusion method with horse antihuman gamma-globulin serum in this group. None showed levels below the normal for this method. This is the extent of our investigation for additional evidence of an impaired immune response. These tests thus far have not provided evidence that might explain the prolonged persistence of skin homografts in uremic recipients.

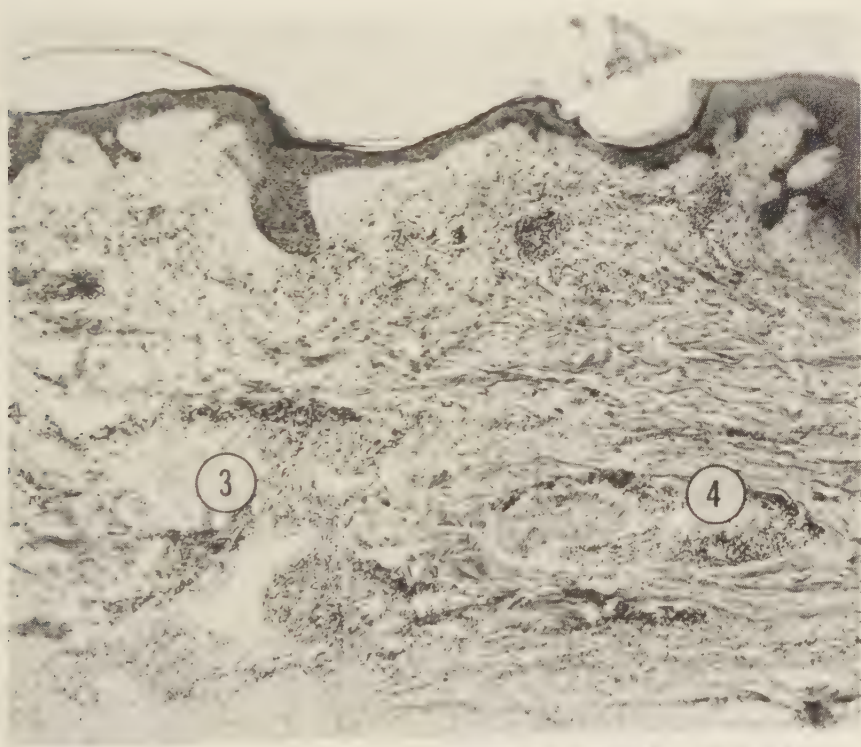


FIGURE 3. Homograft of recipient J.F. with perivascular (3), smooth muscle (4), and superficial dermal infiltration with cells, predominantly lymphocytes. Hematoxylin and eosin stain. $\times 100$.

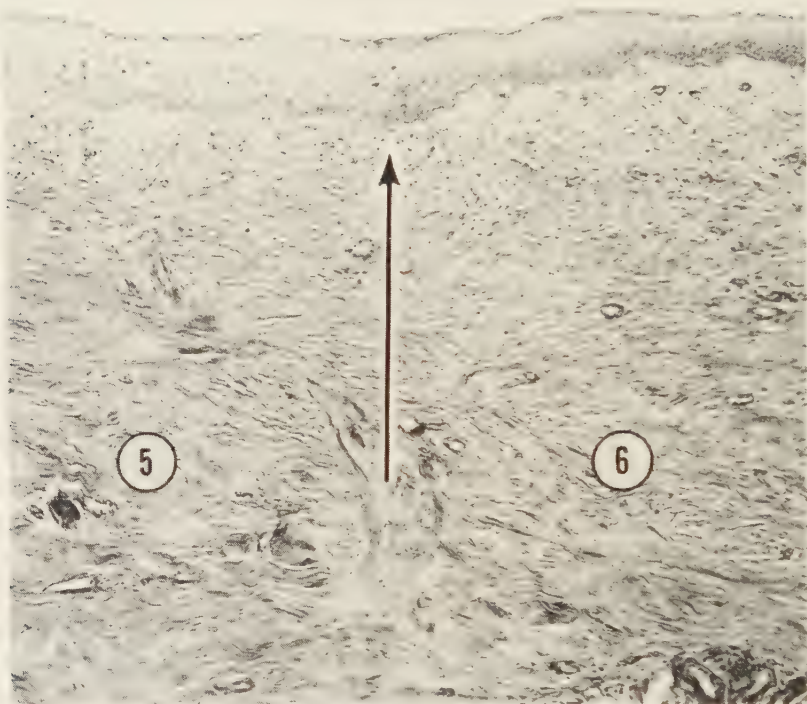


FIGURE 4. Junction of normal skin (6) with normal subepidermal PAS-positive mucopolysaccharide to the right of the arrow and the absence of the same to the left of the arrow in a partially healed granulation tissue (5). Periodic acid-Schiff (PAS) stain. $\times 115$.

Discussion

In those patients studied by Hume *et al.*,¹ who manifested prolonged functional survival of renal homotransplants, there was evidence in the homotransplant itself that this was not the usual morphologic pattern of rejection. The manner in which the recipient epidermis proliferated over the homotransplant ureter provided the epithelial surface for the distal portion of the ureter (FIGURE 7). The persistence of smooth muscle in the wall of the ureter and the media of the arteries, the persistence and function of many nephrons, notably the tubular portions (FIGURE 8), and the prolonged functional survival itself all represent a high degree of receptivity, particularly since these features were observed in a renal homotransplant that functioned for almost 6 months (case No. 9 G.W. from Hume *et al.*¹). The morphologic features in this case that did resemble those of the rejection pattern in the dog homograft included the perivascular and periglomerular cellular infiltrate consisting mainly of lymphocytes and plasma cells, the latter showing cytoplasmic pyroninophilia and the persistence of glomeruli of essentially normal appearance. The morphologic alterations in the dog renal homograft, observable when the first evidence of

insufficiency appears, consist of cellular infiltration and tubular degeneration. Similar changes were observed in the case of Michon *et al.*² Darmady *et al.*³ have described a "narrowing of the glomerular tubular neck with epithelial atrophy" in the microdissection of homotransplanted dog kidneys. Prolonged persistence of the tubular portions of the nephron characterized the renal homotransplant with the longest period of function in the uremic recipient, as mentioned above. In the chronic uremic recipient several components of the kidney homotransplant persisted and functioned; from the data above, the same phenomenon is shown by the skin in such a recipient.

Our studies thus far have not defined the basis for the prolonged persistence of skin homografts in uremic recipients. These patients have been observed to have normal isoagglutinin titers, normal responses to blood group-specific substances, normal paper electrophoretic patterns for antibody proteins, and normal gamma-globulin levels. Furthermore, they may have negative Schick tests and positive tuberculin tests, and they are capable of a plasma cell response. We have not determined complement levels but, from the work of others who have studied complement titers in chronic renal insufficiency, such levels have been found to be normal or somewhat elevated.¹ In patients with

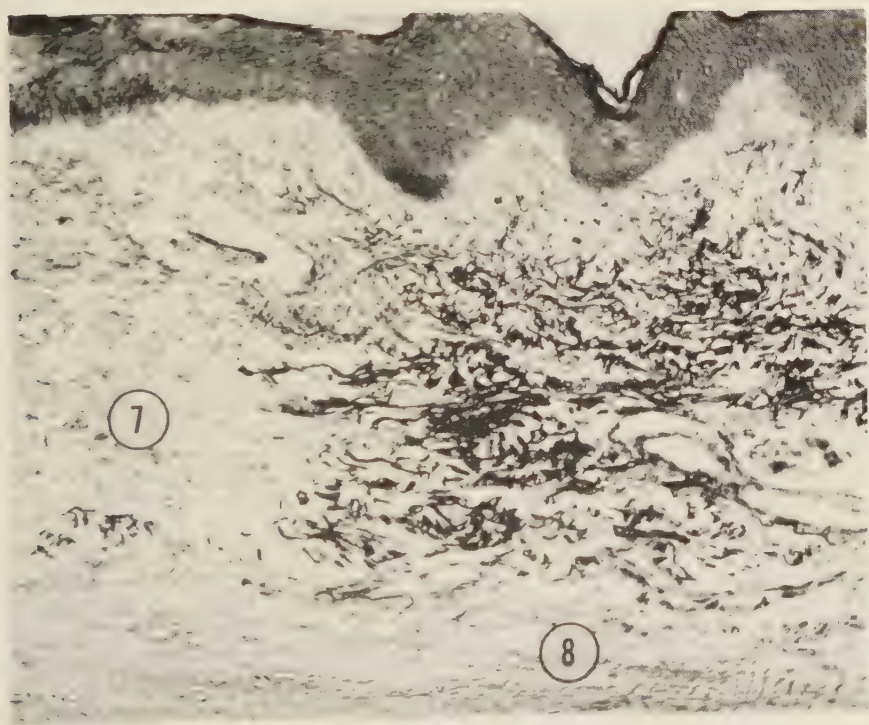


FIGURE 5. Recipient D.B. (115 days). A homograft with an essentially normal epidermis and an elastic fiber pattern is in the upper right portion of the field. Note the absence of elastic fibers in the junction on the left (7) and below (8). Verhoeff-van Gieson stain. $\times 175$.

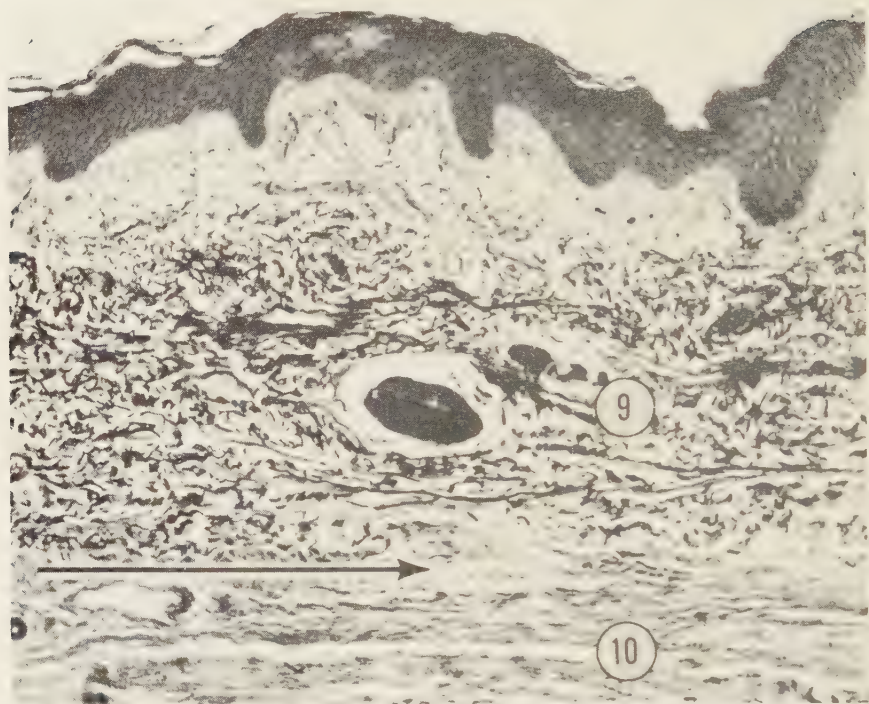


FIGURE 6. Recipient D.B. (115 days), showing the homograft (9) with an essentially normal epidermis, an elastic fiber pattern, and smooth muscle. Compare this with the normal skin shown in FIGURE 1. The arrow denotes the margin between the homograft (9) and the collagenous junction (10) which, as in FIGURE 5, lacks elastic fibers. Verhoeff-van Gieson stain. $\times 175$.

acute renal insufficiency Balch¹⁰ observed normal complement levels and found no impairment of the immune response to tetanus toxoid. Proneness to infection, observed in both chronic and acute renal insufficiency cannot, on the basis of the measurements used and obtained thus far, be related to an impairment of the immune response. No data on properdin levels in chronic renal insufficiency are available at this time. A case observed by Hinz¹¹ suggests that properdin may be involved in the phenomenon of prolonged survival of homograft tissue in chronic uremia. This patient, with glomerulonephritis, during a period of prolonged uremia, had depressed properdin levels that rose as the renal function improved and nitrogen retention was corrected.

Prolonged survival of skin homografts has also been observed in patients with agammaglobulinemia¹² and in patients with extensive burns.¹³ In the agammaglobulinemic, group-O boy studied by Good and Varco¹² there was a continued survival of a skin homograft from a group-A adult female donor. Although such patients have little or no circulating gamma globulin, they are apparently capable of having positive tuberculin tests, as described by Porter.¹⁴ The basis for the prolonged survival of skin homografts in some patients with

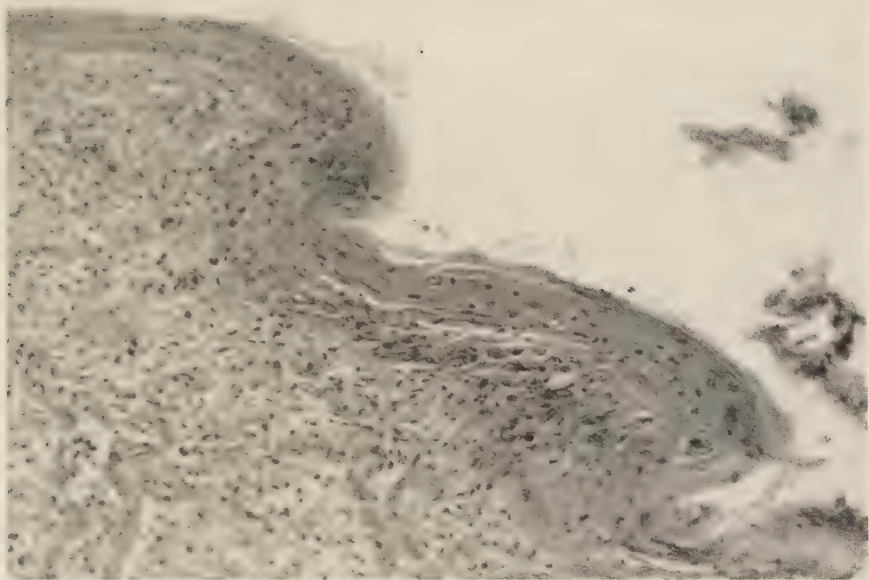


FIGURE 7. Ureter of a renal homotransplant, with the surface of stratified squamous epithelium from recipient G.W. Hematoxylin and eosin stain. $\times 200$.

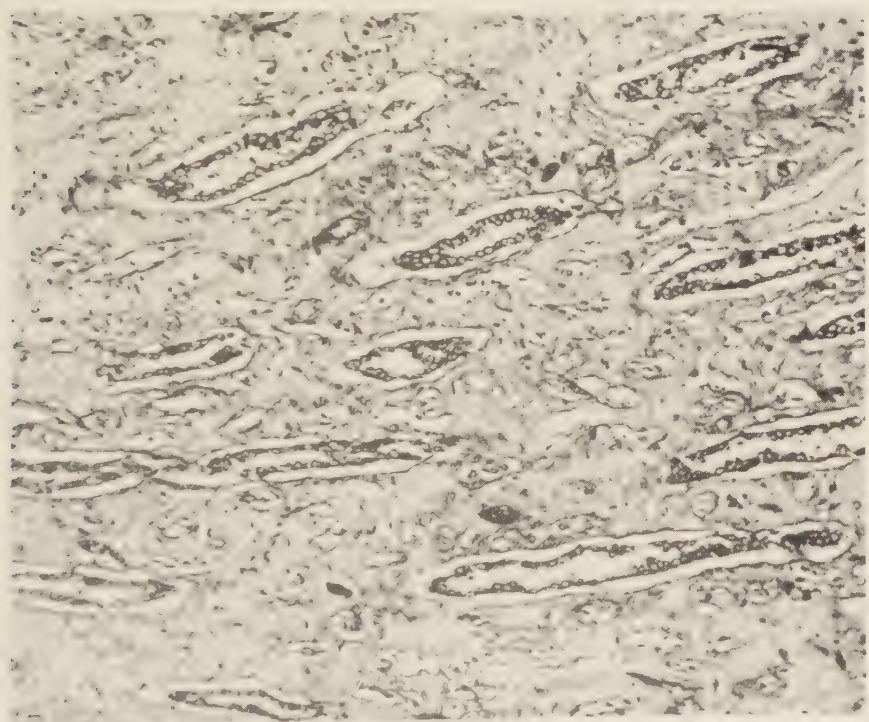


FIGURE 8. Medulla of the renal homotransplant of recipient G.W., showing the collecting tubules with an essentially normal epithelium and interspersed atrophic tubules. Verhoeff-van Gieson stain. $\times 175$.

extensive burns is not known exactly, but such patients are known to manifest prolonged adrenal-cortical stimulation,¹⁵ accelerated protein catabolism, and massive amino-acid excretion,¹⁶ and it is possible that the immune response may be altered under these conditions.

Summary

Prolonged survival of skin homografts has been observed in recipients with chronic uremia. This might be expected, since renal homotransplants have functioned longer in recipients with chronic uremia, and because in mammalian species studied by transplantation procedures thus far, there is an antigenic relationship between the skin and the kidney. If the basis for the rejection of homografted tissue is an immune response on the part of the recipient, then prolonged survival of such tissue in chronic uremia suggests an impaired immune response. Our studies thus far have not elucidated the nature of this suggested impairment of the immune response, since we have observed normal isoagglutinin titers, normal responses to blood group-specific substances, normal paper electrophoretic patterns for antibody proteins, and normal gamma-globulin levels in chronic uremia. Such patients have also had negative Schick tests and positive tuberculin tests. The possibility that the properdin system, the combination of antigen and antibody, and the response to antigen-antibody complexes may be altered in chronic uremia will be studied.

Acknowledgments

The authors acknowledge the generous assistance of Fred H. Allen, Louis K. Diamond, and David Gitlin with the serologic studies, and of John P. Merrill with the clinical studies.

References

1. HUME, D. M., J. P. MERRILL, B. F. MILLER & G. W. THORN. 1955. *J. Clin. Invest.* **34**: 327.
2. MICHON, L., J. HAMBURGER, N. OECONOMOS, P. DELINOTTE, G. RICHET, J. VAYSSE & B. ANTOINE. 1953. *Presse méd.* **61**: 1419.
3. DEMPSTER, W. J. 1953. *Brit. J. Plastic Surg.* **5**: 228.
4. SIMONSEN, M. 1953. *Acta Pathol. Microbiol. Scand.* **32**: 36.
5. MERRILL, J. P., J. E. MURRAY, J. H. HARRISON & W. R. GUILD. 1956. *J. Am. Med. Assoc.* **160**: 277.
6. CONVERSE, J. M. & F. T. RAPAPORT. 1956. *Ann. Surg.* **143**: 306.
7. ELLISON, E. H., B. C. MARTIN, R. D. WILLIAMS, H. W. CLATWORTHY, G. HAMWI & R. M. ZOLLINGER. 1951. *Ann. Surg.* **134**: 495.
8. MOORE, K. L., M. A. GRAHAM & M. L. BARRE. 1953. *Surg. Gynecol. Obstet.* **96**: 641.
9. DARMADY, E. M., W. J. DEMPSTER & F. STRANACK. 1955. *J. Pathol. Bacteriol.* **70**: 225.
10. BALCH, H. H. 1955. *Ann. Surg.* **142**: 145.
11. HINZ, C. F., JR. Personal communication.
12. GOOD, R. A. & R. L. VARCO. 1955. *J. Am. Med. Assoc.* **157**: 713.
13. BROWN, J. B. 1955. *Intern. Abstr. Surg.* **101**: 209.
14. PORTER, H. M. 1957. *Ann. N. Y. Acad. Sci.* **64**(5): 932.
15. HUME, D. M., D. H. NELSON & D. W. MILLER. 1956. *Ann. Surg.* **143**: 316.
16. EADES, C. H., JR., R. L. POLLACK & J. D. HARDY. 1955. *J. Clin. Invest.* **34**: 1756.

THE SURVIVAL OF HOMOGRAFTS IN MICE PRETREATED WITH ANTISERA TO MOUSE TISSUE*

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Introduction

The investigations reported here, made by my colleagues and myself stem historically from early attempts to develop an immune therapy for cancer. An experimental approach to this goal appeared to be offered by the demonstration that some cancers could be transplanted from animal to animal of the same species to which the cancers were indigenous. Anomalous results, however, were soon reported by a number of investigators, who found that tumor grafts sometimes grew better in seemingly immunized animals when the reverse should have been expected. Thus, as early as 1907, Flexner and Jobling,¹ found that a second graft of a transplantable rat sarcoma grew progressively in a large proportion of rats in which a first graft of the same tumor had previously regressed. In the early 1900's there also appeared several papers in which it was reported that tumors implanted in mice or rats pretreated with killed tumor tissues, prepared in a variety of ways, grew more successfully than in untreated controls (for a review of the early literature, see Snell *et al.*²).

The first systematic studies on this phenomenon were done by Casey and his co-workers,³⁻⁷ who used the Brown-Pearce tumor in rabbits and two transplantable mouse tumors in mice.^{8, 9} These investigators found that grafts of the Brown-Pearce tumor grew and metastasized more rapidly and more widely in rabbits pretreated with killed homologous tumor tissues. They also demonstrated that the effect was specific in that it could be produced only by killed tumor tissue derived from the transplantable tumor under study.^{5, 7, 8, 9} Later, Snell and his co-workers,^{2, 10} using a variety of inbred strains of mice and transplantable tumors, established the presence of the phenomenon in animals pretreated with freeze-dried tumor tissue, and demonstrated that the effect was obtained with some host-graft combinations and not with others.

All these observations raised the question of whether the phenomenon was due to specific cancerous substances that behaved as "growth-stimulating" factors for cancers, or whether there was involved some more general principle that was an expression of experimentally altered host-graft relationships. My colleagues and I addressed ourselves to this question and demonstrated that homografts of transplantable tumors would survive in mice pretreated with normal mouse spleen, kidney, or liver.¹¹⁻¹³

It was also found,¹⁴ with a host-tumor combination in which the grafts normally survived in about 50 per cent of the untreated mice, that very small doses of freeze-dried homologous tumor tissue would immunize a significant proportion of the mice to a subsequent tumor graft, while larger doses led to an

* The work reported here was supported in part by a grant in aid from the American Cancer Society, New York, N. Y., upon recommendation of the Committee on Growth of the National Research Council, Washington, D. C., and in part by a research grant (C-1594) from the National Cancer Institute of the National Institutes of Health, Public Health Service, Bethesda, Md.

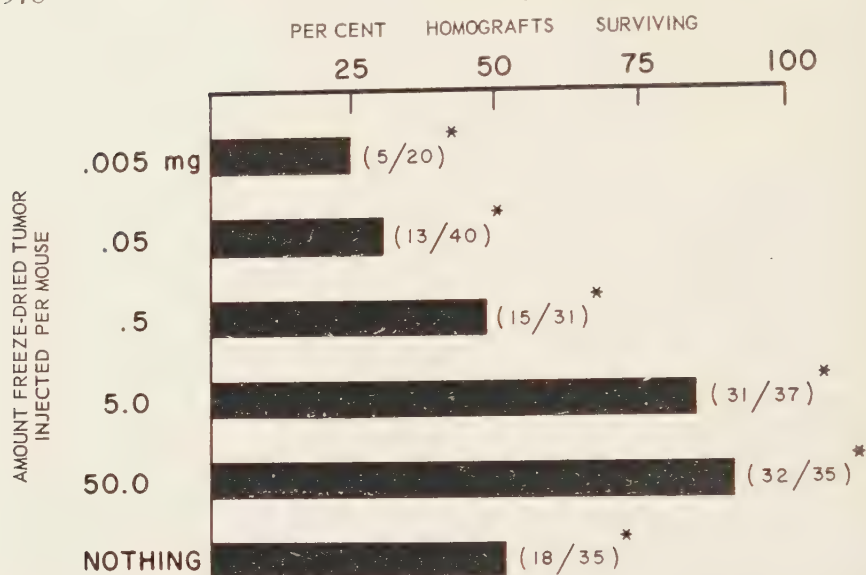


FIGURE 1. The incidence of survival of homografts (that is, per cent mice dying with tumors) of the tumor E 0771 (indigenous to the C57Black/6 strains of mice) in C3H/Ks mice pretreated with different doses of freeze dried tumor E 0771.

* Numerator: No. mice dying with tumors. Denominator: total No. mice in the group.

abrogation of immunity (FIGURE 1). It was concluded from these data that we were dealing with an immune phenomenon rather than with specific cancer growth-stimulating substances. Further support for this conclusion came from experiments in which the time interval between pretreatment with tissue and the grafting of tumor was varied from 1 day up to 46 weeks.¹⁵ It was found that homografts survived in a significant proportion of the mice that received the grafts as long as 46 weeks after the tissue injections (FIGURE 2). Tissue injected *after* tumor grafting did not lead to acceptance of the grafts.

A further step toward elucidation of the processes underlying the induced survival of tumor homografts was our demonstration that *antisera* to mouse tissues (produced in rabbits or mice), when injected into the prospective host prior to tumor grafting, will insure the survival of the homografts in mice.¹⁶⁻¹⁸ The conclusion that the effect was due to antibody present in these sera was supported by experiments with serum fractions obtained by salt fractionation or zone electrophoresis.¹⁹ The activity of the sera was associated with the globulin fractions, and probably specifically with the gamma-globulin fraction. The present report is concerned with recent studies on the mode of action of the antisera,^{20, 21} and the contribution of these findings to an understanding of the manner in which pretreatment with *tissues* may lead to tumor homograft survival.

Materials and General Methods

The transplantable mouse tumor used was Sarcoma I, which is indigenous to the inbred A strain of mice. The histological properties of the tumor have been described previously.²² It grows progressively in 100 per cent of strain A/Ks

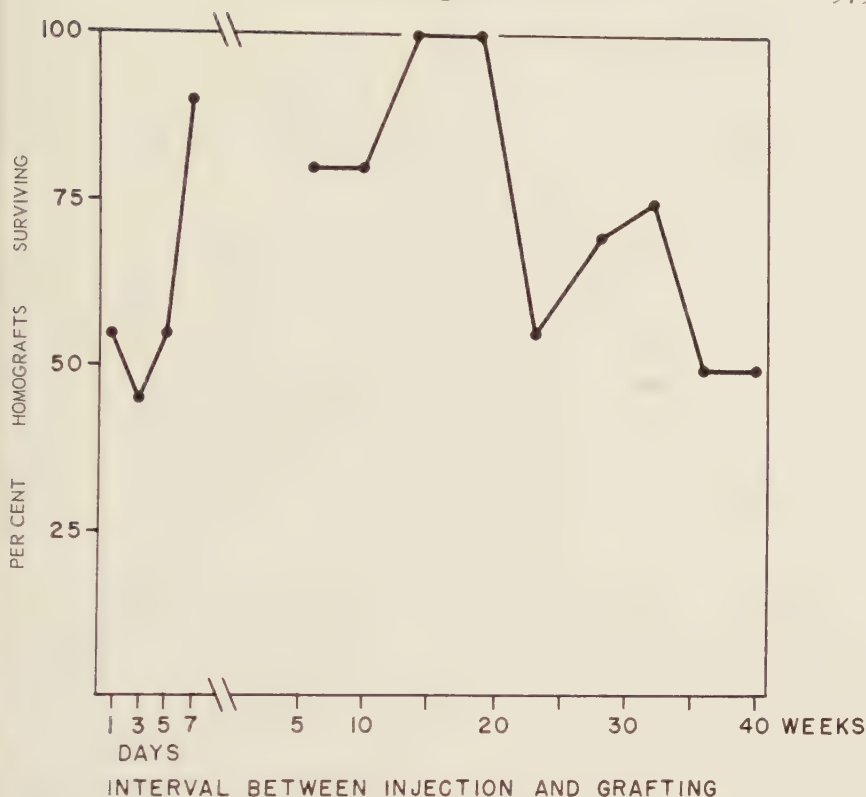


FIGURE 2. The incidence of survival of Sarcoma I grafts (indigenous to the A strain of mice) in C57BL/6Ks mice pretreated with homologous tumor extract at various time intervals prior to tumor grafting.

mice (a strain A subline), killing most of the animals within five weeks after inoculation. Hosts were from the C57BL/6Ks strain (a C57Black subline). Grafts of Sarcoma I very rarely survive in these mice. Characteristically, the grafts grow slightly for about 12 days and then rapidly regress. All mice were 2 to 4 months old at the start of the experiments. Bits of the tumor were inoculated by trocar, under aseptic conditions, in the suprascapular region. The course of growth of the grafts was followed by periodic palpation until the mice either died with a progressively growing tumor or remained without an evident sign of growth for a consecutive period of two months. The latter animals were then classified as "negative."

The antisera were produced in rabbits and C57BL/6Ks mice. The rabbits were immunized with saline homogenates of freshly secured splenic tissue from strain A Ks mice, or with freeze-dried Sarcoma I. The mice were immunized with the supernatants of centrifuged homogenates of previously frozen Sarcoma I. The immunization protocols have been given elsewhere.¹¹ The antisera were administered intraperitoneally to the test mice.

The tissue extracts used for pretreating the mice, to effect homograft survival, were tumor supernatants prepared by high-speed centrifugation of saline

homogenates of Sarcoma I. The details of preparation have been given previously.¹³ The so-called "full-strength" extract was prepared from a 1:1 homogenate (by weight) of tumor and 0.85 per cent NaCl. Dilutions of the "full-strength" supernatant were made to the desired concentrations with sterile 0.85 per cent NaCl. The extracts were administered intraperitoneally to the test mice.

In the experiments with cortisone, all the mice, whether or not they were to receive cortisone, were fed 0.2 per cent terramycin "Animal Formula"* in the drinking water during the full week prior to the start of the experiments. This was done to prevent the activation of latent infections in the cortisone-treated animals. Cortisone acetate (Cortone, Merck)† was injected subcutaneously daily, except Saturdays and Sundays, in the amount of 0.05 ml. per injection. The cortisone was diluted to the desired concentrations, by weight, with "Aqueous Vehicle No. 1" (Merck)†. The administration of cortisone was started on the same day as, or 3 to 4 days before, the first injection of tumor extract. The cortisone was discontinued on the day of, or one day before, grafting of live tumor.

Experiments and Results

Demonstration of the presence of antibody. Experiments with antiserum fractions¹⁹ showed that their activity, that is, the ability to insure homograft survival, was associated with the globulins and most likely with the gamma-globulin fraction. The sera, whether produced in rabbits or mice, were comparatively stable when heated for one hour at 70° C. This is in contrast to the lability of tissue preparations heated at temperatures above 60° C. Other characteristics of the sera, or serum fractions, indicated that their activity could not be ascribed to a substance (or substances) introduced with the tissues used to immunize the rabbits or mice, and possibly recovered in the sera of these animals. The activity of the sera, for example, was lost after absorption with ascites cells of Sarcoma I (unpublished data).

A further demonstration that we were dealing with an antibody was made by a study of the *time relationships* between the injection of antiserum and tumor grafting found necessary to ensure homograft survival. The data are presented in FIGURES 3 and 4. The data of FIGURE 3 are from experiments with rabbit antiserum, and those of FIGURE 4 from experiments with isoantiserum produced in C57BL/6Ks mice.

The data of FIGURES 3 and 4 differ in two ways from those obtained with mice treated with tissues. The effect of the tissue injections persisted for at least 10 months (FIGURE 2) and no homograft survival was induced in the animals receiving the tissue injections *after* tumor grafting. With the antisera, their effect rapidly diminished as the time interval between injection of the serum and subsequent tumor grafting was increased. This diminution of the effect of the sera with time is undoubtedly due to their continuous elimination by the host. These data again demonstrate that the activity of the antisera

* Generously supplied by Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

† Generously supplied by Merck & Co., Inc., Rahway, N. J.

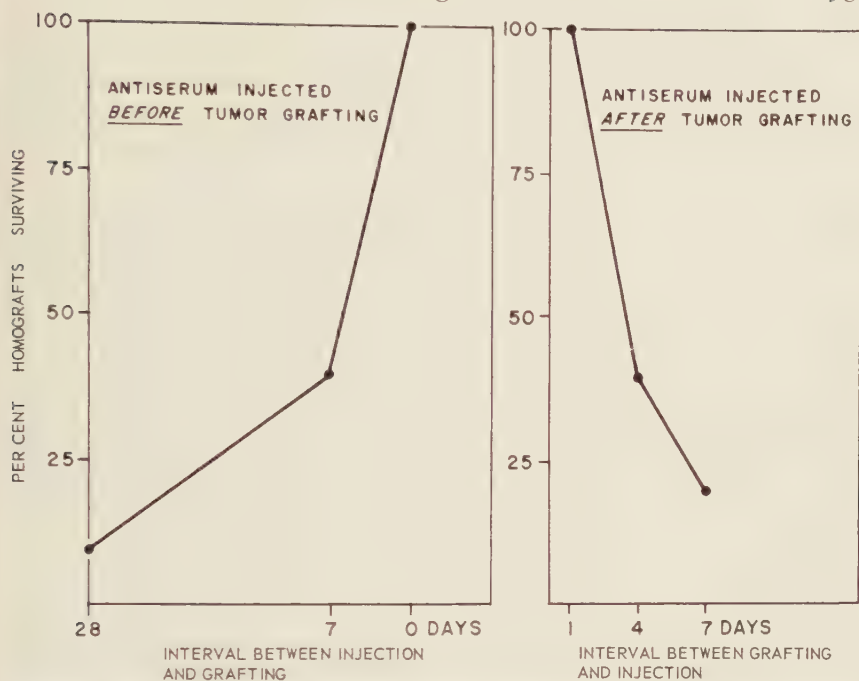


FIGURE 3. The effect of the time of injection of antiserum (produced in rabbits), with respect to the time of tumor grafting, on the incidence of survival of Sarcoma I grafts in C57BL/6Ks mice.

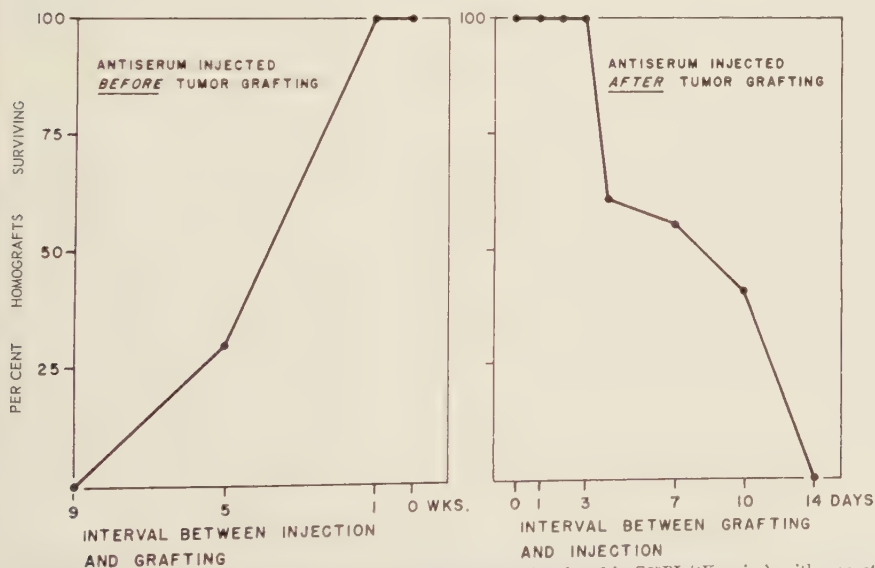


FIGURE 4. The effect of the time of injection of isoantiserum (produced in C57BL/6Ks mice), with respect to time of tumor grafting, on the incidence of survival of Sarcoma I grafts in C57BL/6Ks mice.

is not attributable to the presence of substances introduced by the tissues used for immunization, but rather to "passively transferred" antibody.

Antiserum injected as long as 10 days *after* tumor inoculation (FIGURE 4) insured the survival of a significant proportion of the grafts. The effectiveness of antiserum administered after tumor grafting demonstrates, as we have previously shown,¹⁵ that the grafts may remain viable for at least 10 days, and perhaps longer, in the "foreign" host. These findings also have a bearing upon the mode of action of the antiserum, which will be discussed in detail below.

Another finding in these experiments was the marked growth of the grafts before final regression, as compared with controls, in some of the groups of mice. This reaction can be attributed to the presence of subminimal doses of effective antibody in the mice at the time of tumor grafting, since it was observed chiefly in the groups with the longer time intervals between the administration of the antiserum and the subsequent grafting of tumor.

Demonstration that the presence of antiserum is necessary for homograft survival. The course of production of the antiserum was followed in C57BL 6Ks mice

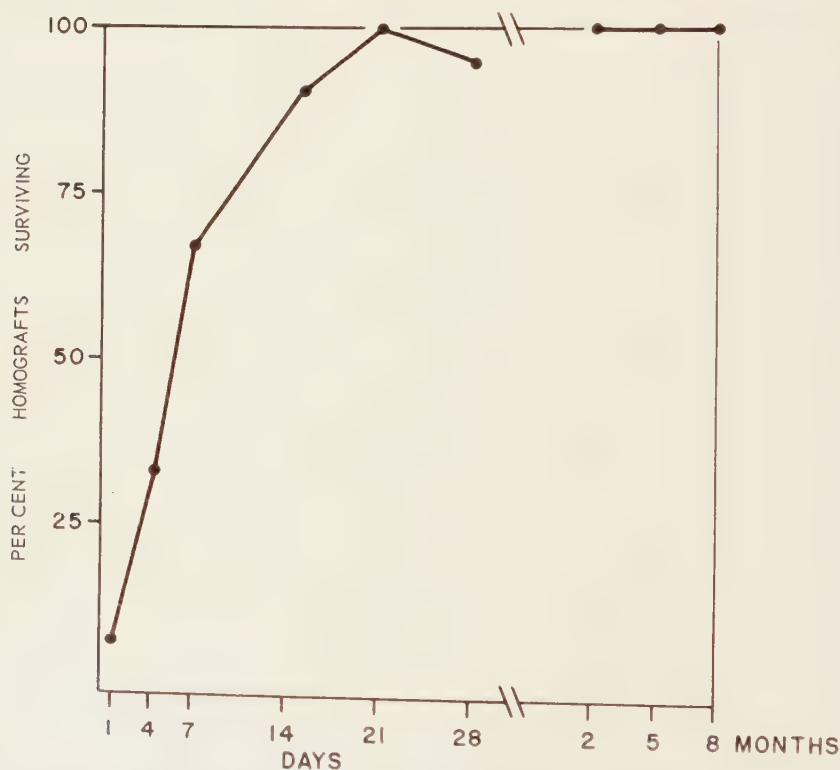


FIGURE 5. Increase in "titer," with time, of isoantiserum effecting tumor homograft survival. The measurement of the "titer" is the incidence of homograft survival, that is, the number of mice dying with progressively growing grafts after receiving passively transferred antiserum. The antisera were obtained by successive bleedings of mice (at the time intervals shown on the horizontal axis) immunized with an inoculum of Sarcoma I.

immunized with either a single inoculum of live Sarcoma I, which grew slightly and then subsequently regressed, or a single intraperitoneal injection of approximately 44 mg. dry weight of Sarcoma I supernatant. The animals were then bled at successive intervals, and the antisera thus obtained were tested for their effect on homograft survival when passively transferred to other C57BL/6Ks mice. FIGURE 5 shows the results obtained with antisera derived from the mice receiving the live tumor inocula. The results obtained with the sera from the mice receiving the tumor supernatant closely parallel these data. Maximum effectiveness was reached with sera obtained at three weeks or longer after immunization (tumor inoculation). Of interest is the fact that an active serum was obtained as long as 8 months after immunization. This observation explains the long persistence of the effects of the tissue injections (FIGURE 2), and supports the conclusion that the production of antiserum in the mice so treated is the decisive factor in ensuring homograft survival.

Effect of cortisone. We had previously shown that homografts of the tumor Sarcoma I will not survive in C57BL 6Ks mice pretreated with certain concentrations of tumor supernatant and cortisone.¹⁵ FIGURE 6 shows the combined data for several experiments, including those first reported,¹⁵ and additional confirmatory data. These data show that cortisone exerts little or no effect at the highest dosage level of tumor supernatant, that the effect of cortisone is evident at supernatant dosage levels of 1:10 and 1:100, and that there is a significant drop in the incidence of surviving tumor homografts in the mice so treated. In the groups of mice receiving cortisone and the lowest dosage of supernatant (1:1000), or cortisone alone, there was a marked transient growth of the grafts before regression.

These findings are explicable on the assumption that the production of an antiserum by the mice receiving the supernatant is a necessary condition for the

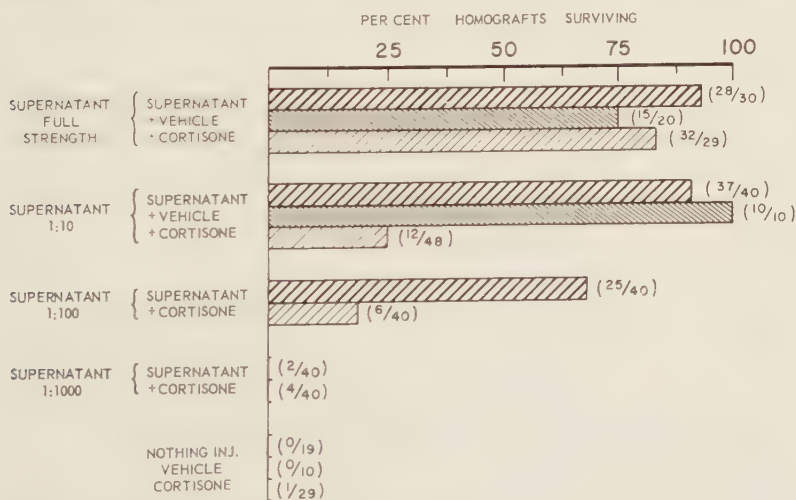


FIGURE 6. The effect of the injection of cortisone during the time of pretreatment with tumor extract on the incidence of survival of Sarcoma I grafts in C57BL/6Ks mice. Numbers in parentheses indicate: No. dying/total No. mice.

survival of the homografts. The assumption is consistent with the report by Hayes and Dougherty,²³ and by Fagraeus (personal communication) that there is a positive correlation between the quantity of antigen administered and the quantity of cortisone necessary to suppress antibody production. To return to the data given in FIGURE 6, we should conclude that the suppressive effect of cortisone on antibody production took place chiefly at supernatant (antigen) dosage levels of 1:10 and 1:100, with the result that the homografts did not survive. Furthermore, it is possible that the suppression by cortisone of the antibody necessary for homograft survival is persistent. We have previously shown that this persistence is true for isohemagglutinin production, and that the suppression may be evident for several months after the initial administration of cortisone and antigen.²⁴

On the other hand, the recovery of the *lymphoid tissue* from the known inhibitory effects of cortisone²⁵ must be fairly rapid, since it is evident that the "homograft reaction" (which appears to be mediated by the lymph nodes²⁶ and perhaps by other tissues) *did take place*, with a consequent destruction of the graft. This assumption of the sequence of reactions in the lymphoid tissue is given credence by the observed larger transient growth of the grafts, as compared with untreated controls, in the mice receiving cortisone alone, or together with the 1:1000 dilution of supernatant. It is emphasized that the administration of cortisone was started on the same day as, or several days before, the start of the supernatant injections, and was stopped on the day of tumor grafting.

Further support for our conclusions is given by experiments in which antiserum was administered to mice that had received injections of tumor supernatant and cortisone at levels that would be expected to lower the incidence of homograft survival (FIGURE 6). The data are shown in FIGURE 7. It is clear that the "passively transferred" antiserum supplied the necessary conditions for homograft survival.

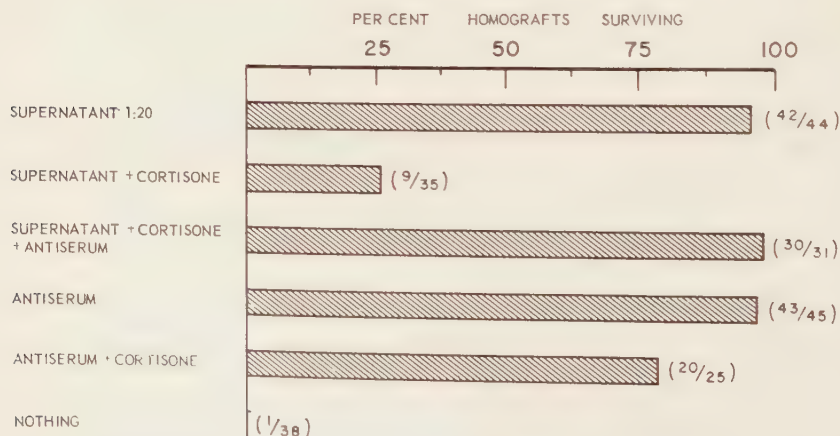


FIGURE 7. The effect of the injection of antiserum on the incidence of survival of Sarcoma I grafts in C57BL/6Ks mice previously treated with tumor extract and cortisone.

The possible mode of action of the antiserum. To study the course of the immune response to the grafts, experiments were carried out with 2 successive inoculations of Sarcoma I in the same host. This procedure was used since it had been shown that mice receiving a tumor inoculum will eventually produce an antiserum that ensures homograft survival (FIGURE 7). We had previously shown that a second graft of Sarcoma I, inoculated at intervals of 1 to 5 months after the mice had received a first inoculum that eventually regressed, will grow progressively.²⁷ We now reduced the intervals between graftings to 1, 2, 3, or 4 weeks and, in some of the groups of mice, antiserum was injected intraperitoneally at the time of the second grafting. The successive grafts were placed subcutaneously in the opposite flanks. FIGURE 8 presents the data of these experiments.

It is apparent that an immune state was evoked by the *first* graft that was not fully overcome by the injection of antiserum, administered along with the *second* graft, for several weeks after the time of inoculation of the first graft. The data for the mice that did not receive antiserum with the second graft show that the immune response begins to wane by the third week after inoculation of the first graft. This coincides with the time when maximum production

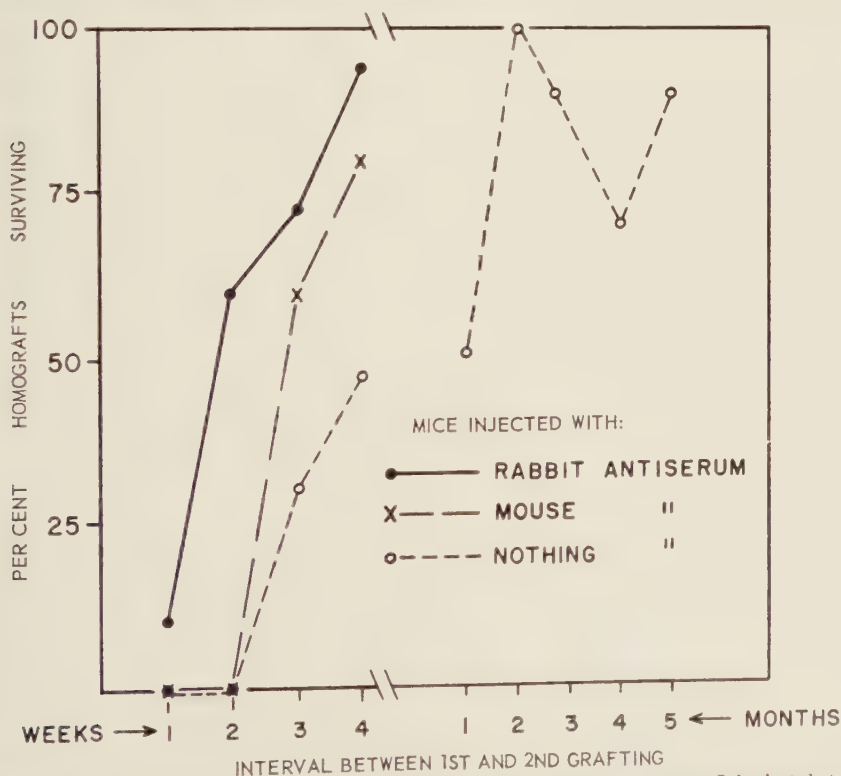


FIGURE 8. The incidence of survival, in C57BL/6Ks mice, of a second graft of Sarcoma I, implanted at increasing time intervals after the first grafting of homologous tumor. Antiserum produced in rabbits or C57BL/6Ks mice was administered to some of the groups of mice at the time of the second grafting.

of the antiserum effecting homograft survival is reached (FIGURE 7). Another evidence of the immune state induced by the first graft, which was apparent in the groups of mice *not* receiving antiserum with the second inoculum, was the very small growth of the *second* graft before regression, or a complete lack of growth. This is in contrast to the pattern of growth of the *first* graft in the same mice, or the usual picture in untreated mice receiving only one graft. As a rule, these grafts grew in size for about 2 weeks after inoculation and then rapidly regressed.

A finding of these experiments, not brought out by FIGURE 8, was that in several instances in which the *first* graft grew progressively, the second graft did or did not survive. Characteristically, in these cases the initial growth of the first grafts was followed by a diminution in size, sometimes to the point where nothing was palpable, and then by a resumption of growth that first became apparent about 2 weeks after the second graft had been inoculated.

Discussion

Our data demonstrate that antiserum produced in mice in response to the tissue injections supplies the necessary conditions for survival of the tumor homografts. (Although the effects of the antisera produced either in rabbits or mice are the same, the possibility that there are qualitative differences in their mode of action is not ruled out.)

Several alternative hypotheses on the role of the antiserum may be considered:

(1) The antiserum "coats" the specific antigens of the tumor inoculum that normally would evoke a "homograft reaction" in the host. Such coated antigens presumably would not stimulate the tissues of the host (lymph nodes,²⁶ and possibly other tissues) that otherwise would react to destroy the graft.

(2) The antiserum acts directly on such reactive centers as lymph nodes to block the "homograft reaction," whether or not this may have already developed in response to a tumor inoculum.

(3) The tumor homograft, coated by the antiserum, is protected from attack by the reactive tissues of the host, even though the mouse has developed an immune response to the inoculum.

(4) The antiserum acts directly on the graft, in some manner thereby altering its responses to the host so that the graft can now survive in an otherwise hostile environment.

We will consider these possibilities in turn. First, the antigens of the tumor homograft, coated by the antiserum, will not evoke the "homograft reaction" of the host. This hypothesis is untenable in view of our findings that passively transferred antiserum injected as long as 4, 7, or 10 days *after* tumor inoculation led to survival of the grafts. That these time intervals should be sufficient to develop the immune responses of the host has been shown by Mitchison,²⁶ who demonstrated that immunity to a tumor homograft could be transferred passively by lymph nodes taken from mice 3 to 10 days after the donors had been exposed to the immunizing stimulus of a tumor homograft inoculum.

The second hypothesis states that the antiserum acts directly upon the reactive centers of the host to block their reaction to the graft. The data from

the experiments with 2 successive tumor inoculations militate against this hypothesis. These data show that, if the interval between the 2 graftings was 2 weeks or less, the immune reaction engendered by the first graft in most instances could not be overcome by the antiserum administered along with the second graft. (These data, incidentally, are in accord with Mitchison's findings²⁶ that passively transferred lymph nodes failed to confer immunity if they were taken from mice more than 10 to 15 days after the immunizing graft had been implanted in the donors.) The seeming contradiction between our data with the double tumor inoculations and the finding that, in mice receiving a single tumor inoculum, antiserum injected at 4, 7, or 10 days after grafting *will* ensure graft survival, will be considered in detail below.

The third hypothesis states that the homograft coated by the antiserum is protected from attack by the previously sensitized reactive tissues of the host. The objections to this hypothesis are the same as those outlined for the second hypothesis.

The fourth hypothesis states that the antiserum in some manner alters the nature of the graft so that it can survive in what otherwise would be a hostile environment. Presumptive support for this premise is afforded by the reports of Barrett and Deringer,^{28, 29} and our own unpublished data indicating that a tumor homograft that survives in mice of a "foreign" strain loses some of its specificity, since it will grow progressively thereafter when implanted in mice in which it normally would be rejected. Barrett and Deringer used a transplantable tumor that is indigenous to, and will grow in, 100 per cent of C3H mice, but will not grow in strain C mice. This tumor was first implanted in F_1 ($C3H \times C$) mice, in which the grafts grew progressively, and tumor derived from these " F_1 " growths was then inoculated into backcross ($F_1 \times C$) mice. Three times as many backcross mice receiving the latter grafts died with progressively growing tumors, as compared with backcross mice receiving grafts directly from C3H donors. It was further determined that the increased transplantability induced by the single passage through a "foreign" (in this case, F_1) host was permanent. To quote Barrett and Deringer:²⁹ "for the moment it is best to describe [the change] more loosely as an induced adaptation in the tumor."

In our own case the altered tumor grafts came from a strain of mice in which their survival had been induced by pretreating the hosts with freeze-dried tumor tissue. Tumors derived from these growths then grew progressively in untreated mice of a strain that normally rejects the grafts. The ability to survive in the foreign host was lost, however, after 7 transplant generations. The reason for this loss is not known.

Casey, Laster, and Ross,³⁰ and Casey and Gunn³¹ have reported an accelerated tumor growth in mice pretreated with killed tumor tissue, even in animals of the strain to which the tumor is indigenous. This characteristic persisted in subsequent transplant generations in untreated mice. Whether or not these examples are pertinent to our argument would depend upon the demonstration of the production of antiserum under the experimental conditions used by these investigators and upon the effects of such an antiserum (if it is produced) upon the growth properties of the tumor grafts.

The marked transient growth of grafts in mice presumed to contain subminimal doses of antiserum at the time of tumor inoculation (see description of experiments relating to FIGURES 3 and 4) may also be attributable to changes in characteristics of the tumor induced by exposure to the antiserum. It may be assumed in these cases that only a small proportion of the tumor cells in each inoculum was affected, and that the number affected was insufficient to ensure continued growth of the graft.

The data of the experiments with double tumor inoculations raise some questions as to the sequence of reactions that may take place both in the host and in the tumor homograft. It is recalled that the immune state induced in a host by the first tumor graft could not be overcome by a second graft implanted 1 or 2 weeks after the first grafting, even though antiserum was administered to the host along with the second graft. On the basis of our previous experience with the effects of passively transferred antiserum, it might have been expected that both the first and second grafts should have grown progressively in the groups receiving the implants a week apart, and that at least the second graft should have survived in the mice receiving the implants 2 weeks apart. Nevertheless, the second graft did not survive, while in several instances it was the *first* graft which resumed progressive growth after the antiserum had been injected.

These results would indicate that two series of events are initiated in the host in response to the first inoculum. One set of events is expressed as the "homograft reaction," which is mediated through the lymph nodes²⁶ (and perhaps other tissues, also) and leads to the destruction of the graft. The second set of events is expressed in the production of the type of antiserum that ensures homograft survival. Such a sequence of reactions has been demonstrated in another fashion by Mitchison and Dube,³² who showed that the hemagglutinating antibody, elaborated by the lymph nodes of a mouse exposed to a tumor homograft, approaches a maximum titer at about 15 to 20 days, by which time the ability of such nodes passively to transfer immunity to a tumor homograft has virtually disappeared.

Our data with the double tumor inoculations indicate that the graft itself undergoes some change in its ability to respond to the passively transferred antiserum during its sojourn in the foreign host. This is shown by those instances in which the *first* graft resumed progressive growth after the antiserum injection, while the second graft did not. This assumption is also supported by the occasional finding that an animal pretreated with tissue will remain "negative" for as long as one month, no graft being palpable during this time, with a subsequent reappearance and progressive growth of the graft. In fact, it is the latter observation which initially led us to carry out the previously reported experiments with double tumor inoculations.²⁷

Finally, it is emphasized that a definitive answer as to the mode of action of the antiserum and to the whole problem of the host-graft relationships we are studying very probably involves processes as yet unknown and perhaps other than those postulated in this paper.

The extent to which the phenomenon is or is not a general one is yet to be established. Is the production of an antiserum a necessary precondition for

tumor homograft survival in other host-graft combinations in rodents, in which it has been demonstrated that pretreatment of the host with tissues leads to an abrogation of resistance to the graft? Furthermore, are our techniques applicable to homografts of normal tissues, or are we dealing with a phenomenon specific to tumors? Others have stated that transplantable tumors may be a poor tool for the study of the homograft reaction as it affects normal tissue grafts, since tumors, once given a slightly favorable handicap in the "struggle for survival," can override and obscure the immune reactions of the host. However, this very characteristic of the tumors, if the case is being correctly stated, may be of fundamental importance to an understanding of cancer biology. The answers to these questions will, of course, come only from further experimentation.

We have been studying the effects of our experimental techniques on homografts of normal tissues of mice—specifically, minced embryos and adult ovary, skin, and spleen. The investigations on ovary grafts are being made in collaboration with Katharine P. Hummel, of our laboratory. The hosts have been pretreated by intraperitoneal administration of extracts of spleen, kidney, or ovary, and with isoantisera to spleen, kidney, and tumor. To date we have had negative results with the adult tissues, and equivocal results with embryo mince. On the other hand there is evidence of immunization in the mice receiving kidney extract and, subsequently, ovary homografts.

In connection with our experiments, the recently reported results of Billingham and Sparrow³³ are of interest. These investigators obtained as much as a threefold increase in survival time of skin homografts in rabbits pretreated by the intravenous route with viable epidermal cells or blood cells of the presumptive donor. The processes underlying these findings are still to be elucidated.

Acknowledgments

It is a pleasure to acknowledge the helpful discussions with Henry Winn, concerning the implications of our data. I am particularly indebted to Bradley F. Bryant for his competent assistance in the conduct of the experiments. I also wish to thank Philip Montella, of our art staff, for the preparation of the figures.

References

1. FLEXNER, S. & J. W. JOBLING. 1907. Restraint and promotion of tumor growth. *Proc. Soc. Exptl. Biol. Med.* **5**: 16-18.
2. SNELL, G. D., A. M. CLOUDMAN, E. FAJOR & P. DOUGLASS. 1946. Inhibition and stimulation of tumor homoiotransplants by prior injections of lyophilized tumor tissue. *J. Natl. Cancer Inst.* **6**: 303-316.
3. CASEY, A. E. 1932. Experimental enhancement of malignancy in the Brown-Pearce rabbit tumor. *Proc. Soc. Exptl. Biol. Med.* **29**: 816-818.
4. CASEY, A. E. 1934. A persistent hypersusceptibility induced in rabbits with an homologous tumor material. *Proc. Soc. Exptl. Biol. Med.* **31**: 666-667.
5. CASEY, A. E. 1934. Specificity of enhancing materials from mammalian tumors. *Proc. Soc. Exptl. Biol. Med.* **31**: 663-665.
6. CASEY, A. E. 1941. Experiments with a material from the Brown Pearce tumor. *Cancer Research*. **1**: 134-135.
7. CASEY, A. E., L. MEYERS & G. R. DRYSDALE. 1948. Selective blocking of host resistance to malignant neoplasm (Brown-Pearce tumor) in New Zealand White rabbits. *Proc. Soc. Exptl. Biol. Med.* **69**: 579-585.

8. CASEY, A. E. 1933. A species limitation of an enhancing material derived from a mammalian tumor. *Proc. Soc. Exptl. Biol. Med.* **30**: 674-677.
9. CASEY, A. E. 1933. Failure of a mouse carcinoma material to enhance a mouse sarcoma. *Proc. Soc. Exptl. Biol. Med.* **30**: 1025-1026.
10. SNELL, G. D., A. M. CLOUDMAN & E. WOODWORTH. 1948. Tumor immunity in mice, induced with lyophilized tissue, as influenced by tumor strain, host strain, source of tissue, and dosage. *Cancer Research*, **8**: 429-437.
11. KALISS, N. & G. D. SNELL. 1951. The effects of injections of lyophilized normal and neoplastic tissues on the growth of tumor homoio-transplants in mice. *Cancer Research*, **11**: 122-126.
12. SNELL, G. D. 1952. Enhancement and inhibition of the growth of tumor homoio-transplants by pretreatment of the hosts with various preparations of normal and tumor tissue. *J. Natl. Cancer Inst.* **13**: 719-729.
13. DAY, E. D., N. KALISS, A. I. ARONSON, B. F. BRYANT, D. FRIENDLY, F. C. GABRIELSON & P. M. SMITH. 1954. Investigations of substances in mouse tissues inducing alteration of normal host-homograft relationships. *J. Natl. Cancer Inst.* **15**: 145-159.
14. KALISS, N. 1952. Regression or survival of tumor homoio-grafts in mice pretreated with injections of lyophilized tissues. *Cancer Research*, **12**: 379-382.
15. KALISS, N. & E. D. DAY. 1954. Relation between time of conditioning of host and survival of tumor homografts in mice. *Proc. Soc. Exptl. Biol. Med.* **86**: 115-117.
16. KALISS, N. & N. MOLOMUT. 1952. The effect of prior injections of tissue antisera on the survival of cancer homoio-grafts in mice. *Cancer Research*, **12**: 110-112.
17. KALISS, N., N. MOLOMUT, J. L. HARRISS & S. D. GAULT. 1953. Effect of previously injected immune serum and tissue on the survival of tumor grafts in mice. *J. Natl. Cancer Inst.* **13**: 847-850.
18. KALISS, N. 1955. Induced alteration of the normal host-graft relationships in homo-transplantation of mouse tumors. *Ann. N. Y. Acad. Sci.* **59**(3): 385-391.
19. KALISS, N. & A. A. KANDUTSCH. 1956. Acceptance of tumor homografts by mice injected with antiserum. I. Activity of serum fractions. *Proc. Soc. Exptl. Biol. Med.* **91**: 118-121.
20. KALISS, N. 1956. Acceptance of tumor homografts by mice injected with antiserum. II. Effect of time of injection. *Proc. Soc. Exptl. Biol. Med.* **91**: 432-437.
21. KALISS, N. 1956. Survival of tumor homografts in mice pretreated with tissue extract, antiserum and cortisone. To be published.
22. KALISS, N., P. R. F. BORGES & E. D. DAY. 1954. The survival and metastatic spread of homografts of mouse tumors in mice pretreated with lyophilized tissue and cortisone. *Cancer Research*, **14**: 210-219.
23. HAYES, S. P. & T. F. DOUGHERTY. 1952. Effect of ACTH and cortisone on antibody synthesis and rate of disappearance of antigen. *Federation Proc.* **11**: 67.
24. KALISS, N., G. HOECKER & B. F. BRYANT. 1956. The effect of cortisone on isohemagglutinin production in mice. *J. Immunol.* **76**: 83-88.
25. EHRLICH, W. E. 1953. The effect of corticosteroids upon lymphoid tissue. *In* The Effect of ACTH and Cortisone upon Infection and Resistance. Columbia Univ. Press, New York, N. Y.
26. MITCHISON, N. A. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. I. The role of lymph node cells in conferring immunity by adoptive transfer. *J. Exptl. Med.* **102**: 157-177.
27. KALISS, N. 1955. Reversal of the "second-set response" in tumor homotransplantation. *Transplantation Bull.* **2**: 52-53.
28. BARRETT, M. K. & M. K. DERINGER. 1952. Induced adaptation in a tumor: permanence of the change. *J. Natl. Cancer Inst.* **12**: 1011-1017.
29. BARRETT, M. K. & M. K. DERINGER. 1950. An induced adaptation in a transplantable tumor of mice. *J. Natl. Cancer Inst.* **11**: 51-59.
30. CASEY, A. E., W. R. LASTER, JR. & G. L. ROSS. 1951. Sustained enhanced growth of carcinoma E 0771 in C57Black mice. *Proc. Soc. Exptl. Biol. Med.* **77**: 358-362.
31. CASEY, A. E. & J. GUNN. 1952. XYZ effect in strain of origin: E 0771 carcinoma in C57BL/6 mice. *Proc. Soc. Exptl. Biol. Med.* **80**: 610-613.
32. MITCHISON, N. A. & O. L. DUBE. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. II. The relation between hemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations. *J. Exptl. Med.* **102**: 179-197.
33. BILLINGHAM, R. E. & E. M. SPARROW. 1955. The effect of prior intravenous injections of dissociated epidermal cells and blood on the survival of skin homografts in rabbits. *J. Embryol. Exptl. Morph.* **3**: 265-285.

Discussion of the Paper

RUPERT BILLINGHAM (*University College, London, England*): The work of Kaliss and Snell and their colleagues on the phenomenon of "enhancement"—or, as Kaliss would prefer to call it, the induced acceptance of a tumor homograft by mice that would have normally rejected it—has commanded wide attention, primarily because of its possible relevance to a solution of the problem of how to transplant homografts of normal tissues with permanent success, but also, I suspect, because, rather provocatively, the underlying mechanism involved in this phenomenon has hitherto remained such a closely guarded secret of Nature.

I think it will be agreed that the paper Kaliss has just presented gives us, apparently for the first time, some insight into the principles that underlie "enhancement"—I shall continue to use this term for the sake of brevity. The essential prerequisite for enhancement appears to be the presence in the intended host, at or near the time of tumor inoculation, of antibodies (antisera) specifically directed against some component of the tumor. These antibodies may be caused to appear in the host in any one of three different ways, with similar results: (1) by active immunization of the host with prior inoculations of frozen-dried tissue or with saline homogenates prepared from the fresh tumor; (2) by the passive transfer of the ready-made antibodies in serum from mice, or even rabbits, that themselves have undergone active immunization; or (3) by the active immunization of the host with a *living* tumor graft.

Kaliss has considered a variety of hypotheses that may account for the ability of these antibodies to procure the acceptance of tumor homografts in otherwise resistant hosts. I propose to consider in more detail the nature of these antibodies and the apparently paradoxical situation whereby the outcome of homotransplanting a tumor may be greatly improved as a consequence of the presence in the host of antibodies specifically directed against it.

With the possible exception of leukemias, it is now generally accepted that transplantation immunity cannot be transferred passively by means of serum from animals that have reacted against living-tissue homografts. To do this, the use of activated lymph-node cells appears to be obligatory. Furthermore, no one has succeeded in demonstrating any adverse influence of putatively immune sera on the structure or viability of potentially susceptible cells *in vitro*. Such facts as these argue very strongly that whatever the mechanism of homograft breakdown may be, the causative agent is not normally present in the serum.

It is a well-established fact, however, that homografts of both tumor and normal tissue do elicit the formation of antibodies that are present in serum and that reveal themselves by their capacity to agglutinate red cells under the appropriate conditions. By definition, these antibodies are hemagglutinins, and their formation is elicited by antigens shared alike by red cells and living tissue cells. The work of Mitchison and Dube shows clearly that these antibodies are also elicited by homologous lyophilized tissues, or by saline homogenates of the fresh tissues. We can be quite certain, I think, that these hemag-

glutinins are the antibodies responsible for enhancement. Certainly, they may be absorbed by living cells, but there is not the slightest evidence that they have any harmful effect when this takes place.

What, then, is the nature of the antigens responsible for transplantation immunity—the *transplantation immunity* antigens, or "histocompatibility" antigens, as opposed to the red-cell type antigens? The studies of Little, Snell, and Gorer show clearly that both types of antigens must be genetically determined. My colleagues, Medawar and Brent, and I have recently succeeded in demonstrating the existence of these transplantation immunity antigens that hitherto had been rather hypothetical entities. These substances are present in the nuclei of cells but not in the cytoplasm, and their lability is such that they are completely inactivated by nearly all procedures that kill living cells. For example, they are destroyed by repeated freezing and thawing, by lyophilization, or by heating to less than 50° C. for 20 minutes. It may be added that none of these treatments destroys the red-cell antigens. There is also evidence that the two types of isoantigens differ profoundly with respect to their biochemical constitution: whereas at least some of the red-cell type antigens are almost certainly mucopolysaccharides, as W. T. J. Morgan's work has shown, we have strong indications that the activity of the transplantation immunity antigens is associated with desoxyribonucleic acid. Two experimentally separable systems of isoantigens are therefore present in living cells: the comparatively stable red-cell antigens and the exceedingly labile transplantation immunity antigens. I have already discussed the antibodies that are formed in response to the red-cell antigens, but have said little about the existence of antibodies formed in response to the transplantation immunity antigens. Unfortunately, little is known about these antibodies—their existence must be inferred. The close analogy that exists between the homograft reaction and sensitization reactions of the delayed type suggests rather strongly that they may be cell-bound and are never present in significant amounts in the serum.

It seems to me that a complete understanding of the mechanism of enhancement will not be achieved until we gain a better knowledge of the two systems of isoantigens present in tissue cells, their interrelationships, and the possible "cross reactions" that may take place with their corresponding antibodies. A possible explanation of enhancement, at which Kaliss has already hinted, is that the isohemagglutinins may be able to combine with and inactivate the transplantation immunity antigens as the latter are liberated from homologous tissue grafts, thus attenuating the antigenic stimulus received by the host's draining lymph nodes.

In conclusion, we now have evidence that enhancement is not a phenomenon specifically associated with tumor homografts, for the expectation of survival of skin homografts transplanted between mice of strains A and CBA can be prolonged by a few days as a consequence of pretreatment of the hosts with lyophilized spleen or kidney tissue from the donor strain. In rabbits, too, it has been found that pretreatment of recipients with fairly massive dosages of their intended donor's lyophilized kidney tissue may increase the lifetime of skin homografts by a factor of 2. The apparently superior results obtained

when tumor homografts are used as the test system are almost certainly attributable to the capacity of a tumor, once it has become established, to override the immunological opposition that develops and that would certainly bring about the breakdown of a skin homograft.

Inasmuch as enhancement depends upon the presence of hemagglutinins in a recipient, in any attempt to employ the phenomenon clinically particular care must be taken in the event that the subject may require subsequent blood transfusions. The possibility of compromising the outcome of future pregnancies in premenopausal women should also be borne in mind. Gorer has previously drawn attention to these risks.

NATHAN KALISS: I wish to say a few words about terminology. The phenomenon I am studying, and apparently similar phenomena that have been studied by others, has been variously termed the "XYZ effect" (Casey), or the "enhancing effect" (Snell). In the past I have referred to the procedure of preparing the host with injections of tissue to accept a tumor homograft as "conditioning" of the host.

The term "enhancement" has historical reasons for its use. Casey first used it to describe the observed accelerated growth and metastasis of the Brown-Pearce tumor in rabbits pretreated with killed homologous tumor tissue. The experimental setup Casey used did lend itself to the description of the events observed as "enhanced growth" of the tumor graft, since the tumor survived and metastasized, to a lesser degree, in untreated rabbits. In my own case, this terminology is hardly applicable, for what is being observed is the induced acceptance of a homograft by mice that normally would reject the graft. What we are studying, therefore, is *graft survival*, not enhanced growth. The growth observed is a property of the tumor per se, and it follows the successful establishment of the tumor inoculum in an environment that normally would be hostile. If we should eventually succeed in establishing the survival of homografts of *normal* tissues by similar experimental procedures, it would hardly be appropriate to speak of such grafts as "enhanced." The term "XYZ factor" is noncommittal as to the processes in operation, but my objection to it is the aura of mysticism it appears to lend to the phenomenon.

I must confess that I have no "handle" for the phenomenon. Perhaps a Latin or Greek scholar among readers of this publication can coin a term that will describe the observations.

IMMUNE PHENOMENA ELICITED BY TRANSPLANTED TUMORS. II. CYTOTOXIC EFFECTS OF IMMUNE GUINEA PIG SERA ON MOUSE CANCER CELLS

By O. N. Rambo, Jr., Roger B. Fuson, and E. J. Eichwald

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Transplants of mouse Sarcoma 37 to the brains of guinea pigs grow relatively well and not infrequently cause death of the host. Growth of the transplant can be prevented or inhibited by a preceding inoculation of the same tumor either to the subcutaneous tissue, the eye, or a different location in the brain. Although, in general, both the eye and the brain are relatively isolated from systemic immune events, this inhibition of tumor growth is weaker after a preceding subcutaneous inoculation than after intracranial or intraocular inoculation.¹ This difference in response to the inoculated mouse tumor has been assumed to be a dosage phenomenon related to the fact that the tumors are quickly absorbed after subcutaneous inoculation,² while they grow for a limited period of time in both eye and brain, thereby providing for a larger dose of foreign tumor tissue to which an immune response can develop.

This report deals with *in vitro* studies of cytotoxic antibodies arising in guinea pigs in response to inoculated mouse tumors. Such antibodies have been demonstrated before by Werder *et al.*,³ Imagawa *et al.*,⁴ and Green,⁵ by inoculating mouse tumors into hosts of a foreign species. Specifically, we wished to determine whether the differences in immune response described above, following use of various sites of inoculation, were paralleled by differences in rate of appearance and titer of cytotoxic antibodies. The specificity of these antibodies, the effect of inoculation of nonviable tissue, and the effect of multiple inoculations also were studied.

Materials and Methods

Strain A Jax mice of both sexes, obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me., and approximately 8 weeks of age, were used as tumor carriers and to test the viability of tumor-cell suspensions.

Guinea pigs of either sex, approximately 10 weeks of age and raised in this laboratory on a commercial diet supplemented with lettuce, received injections of mouse Sarcoma 37, normal mouse liver, and normal mouse spleen. The tumor was inoculated into the anterior chamber of 1 eye, subcutaneously in 1 flank, or into the brain⁶ of the guinea pigs. Fragments of fresh liver and spleen from normal mice were inoculated subcutaneously and in the anterior chambers. Guinea pigs were bled by cardiac puncture on specified days to detect cytotoxic antibodies. The blood from members of each group of animals was pooled and the serum separated.

Tumor-cell preparations for cytotoxic studies were made by aseptically removing and weighing fragments from a carrier mouse. The pieces of tumor

were minced with sterile scissors. The mince was mixed with 4 volumes of normal saline and homogenized for 30 seconds. The 1:5 dilution of cells was filtered through an extra-coarse fritted filter (maximum pore size: $160\ \mu$) and used as a stock suspension for further dilutions. Inoculations of 0.02 ml. of this suspension into the brains of susceptible mice killed all animals in approximately two weeks.

To increase the sensitivity of the suspension, the highest dilution that would kill all inoculated mice (MDL 100) was determined. This was found to be 1:1600, using either isotonic sodium chloride or serum as the diluent (0.02 ml. of this dilution amounts to about 8000 Sarcoma 37 cells).

The cytotoxic activity of an immune serum was measured by its ability to inactivate the 1:1600 tumor-cell suspension after incubation for 10 minutes at 37°C ., whereby the serum served as the diluent of the stock suspension. Dilutions with saline solution and with normal guinea pig serum served as controls.

Susceptible mice were injected intracerebrally with 0.02 ml. of the tumor-cell suspension. The mice were observed daily over a period of 50 days for appearance of tumors at the site of injection. Autopsies were performed to substantiate tumor growth.

In the experiments with nonviable tissue, Sarcoma 37 was alternately frozen at -8°C . and thawed 14 times over a period of 14 days. The treated tissue was tested for viability by intracerebral injection of susceptible mice. Mouse liver was rendered nonviable by alternate freezing at -8°C . and thawing 7 times over a period of 7 days. For multiple injections the nonviable tissues were homogenized, filtered, and stored.

Results

(1) One experiment was designed to test the *rates of appearance* of a cytotoxic effect in the sera of guinea pigs that had received injections of tumor at different body sites.

It is apparent from TABLE 1 that growth of tumor cells was not inhibited by *in vitro* incubation in sera from guinea pigs 5 days following injection of tumor at various body sites. Cytotoxicity first appeared on the tenth day following subcutaneous (90 per cent survival of injected mice) and eye (40 per cent survival) inoculation. No cytotoxic effects were evident on the tenth day following brain inoculation (0 per cent survival). Fifteen days after injection of tumor in the eye and the brain, the serum completely inactivated tumor-cell suspensions (100 per cent survival). The serum from the subcutaneously inoculated guinea pigs did not show an increase in cytotoxic activity over that drawn on the tenth day (90 per cent survival). Mice dying of tumor in the 10- and 15-day groups survived longer than their controls. The sera from all three groups drawn on the 20th day produced 100 per cent inactivation of the tumor cells. No cytotoxic effects were evident in the controls.

(2) A second experiment was designed to compare the *titers* of cytotoxic activity in the sera of guinea pigs inoculated with sarcoma 37 at different body sites.

The data in TABLE 2 indicate that sera drawn 20 days following injection of

TABLE 1
CYTOTOXIC EFFECTS OF GUINEA PIG SERA ON MOUSE SARCOMA 37 CELLS:
RATE OF DEVELOPMENT

Diluents used to prepare 1:1600 tumor-cell suspensions	Bleeding dates of guinea pigs following injections of tumors							
	5th		10th		15th		20th	
	*	†	*	†	*	†	*	†
Pooled serum from guinea pigs receiving injections of tumor in EYES	10/10	(14.6)	6/10	(19.0)	0/10—		0/10—	
Controls: normal guinea pig serum	10/10	(13.7)	10/10	(14.0)	10/10	(15.0)	10/10	(14.3)
Controls: isotonic sodium chlo- ride	10/10	(15.1)	10/10	(14.4)	10/10	(15.6)	10/10	(13.9)
Pooled serum from guinea pigs receiving tumor in BRAIN	10/10	(15.1)	9/9	(17.0)	0/10—		0/9—	
Controls: normal guinea pig serum	6/6	(14.8)	6/6	(14.3)	6/6	(14.5)	6/6	(15.0)
Controls: isotonic sodium chlo- ride	6/6	(15.6)	6/6	(15.6)	6/6	(15.3)	5/5	(15.0)
Pooled serum from guinea pigs receiving tumor SUBCUTA- NEOUSLY	10/10	(17.6)	1/10	(27.0)	1/9	(21.0)	0/10—	
Controls: normal guinea pig serum	6/6	(19.0)	6/6	(14.8)	6/6	(15.1)	6/6	(16.5)
Controls: isotonic sodium chlo- ride	6/6	(19.0)	6/6	(15.1)	6/6	(15.3)	6/6	(15.5)

* Mice dying with growing tumors.

† Average survival time, in days, of animals dying with tumor.

TABLE 2
CYTOTOXIC EFFECTS OF GUINEA PIG SERA ON MOUSE SARCOMA 37:
DIFFERENCES IN TITER

Diluents used to prepare 1:1600 tumor-cell suspensions	Dilutions of pooled sera									
	Undiluted		1:2		1:4		1:8		1:16	
	*	†	*	†	*	†	*	†	*	†
Pooled serum from animals receiving tumor in ANTERIOR CHAMBER OF EYE	0/10		0/10		0/10		0/10		10/10	(15.7)
Pooled serum from animals receiving tumor IN BRAIN	0/10		0/10		0/10		0/10		10/10	(14.5)
Pooled serum from animals receiving tumor SUBCUTANEOUSLY	3/10	(15.3)	6/10	(15.0)	4/10	(14.7)	10/10	(15.0)		
Controls: normal guinea pig serum	40/40	(14.8)								

* Mice dying with growing tumors.

† Average survival time, in days, of mice dying with tumor.

tumor in the eye and the brain had sufficient cytotoxic activity to afford complete protection to mice in dilutions of 1:8. The 1:16 dilutions did not exhibit any cytotoxic properties. Only partial cytotoxicity was exhibited by the serum of guinea pigs that had received subcutaneous injections of tumor (70 per cent survival). Less cytotoxicity was exhibited by 1:2 and 1:4 dilutions of this serum (40 and 60 per cent survival). There was no evidence of protection by the 1:8 dilution. The 40 mice in the control groups died with tumors.

(3) An attempt was made to compare the antigenic properties of *viable* and *nonviable* tumor inoculated subcutaneously and in the eye of guinea pigs.

Twenty days following the injection of killed and living tumor into groups of guinea pigs, the pooled sera were tested for their respective cytotoxic effects on tumor cells. No cytotoxic activity was exhibited by the sera of guinea pigs that had received single injections of nonviable tumor in the eyes and in the subcutaneous tissue (TABLES 3 and 4). As before, complete protection was provided by the sera from the guinea pigs that had received single injections of viable tumor in one eye. A weaker cytotoxic effect was present in the serum of guinea pigs that had received single subcutaneous inoculations of viable tumor (43 per cent survival). All control mice receiving intracerebral injections of tumor cells in normal guinea pig serum died.

(4) A fourth experiment was designed to determine whether inoculation of *normal mouse tissues* into guinea pigs would result in the production of anti-

TABLE 3
CYTOTOXIC EFFECTS OF GUINEA PIG SERA FOLLOWING SUBCUTANEOUS INJECTIONS
OF VIABLE AND NONVIABLE MOUSE SARCOMA 37

Diluents used to prepare 1:1600 tumor-cell suspensions	Mice receiving intracerebral injections of tumor-cell suspensions	
	*	†
Pooled serum from animals receiving nonviable tumor	30/30	13.6
Pooled serum from animals receiving viable tumor	17/30	19.7
Controls: pooled normal guinea pig serum	30/30	13.0

* Mice dying with growing tumors.

† Average survival time, in days, of animals dying with tumor.

TABLE 4
CYTOTOXIC EFFECTS OF GUINEA PIG SERA FOLLOWING EYE INJECTIONS OF VIABLE
AND NONVIABLE MOUSE SARCOMA 37

Diluents used to prepare 1:1600 tumor-cell suspensions	Mice receiving intracerebral injections of tumor-cell suspensions	
	*	†
Pooled serum from animals receiving nonviable tumor:	15/15	16.8
Pooled serum from animals receiving viable tumor	0/15	—
Controls: pooled normal guinea pig serum	15/15	17.0

* Mice dying with growing tumors.

† Average survival time, in days, of animals dying with tumor.

bodies cytotoxic to mouse Sarcoma 37 cells. Of 30 guinea pigs in the subcutaneous group, 10 received fragments of fresh mouse liver, 10 received fresh spleen, and 10 were inoculated with viable tumor. Thirty additional guinea pigs received similar inoculations in the eye.

It is evident from TABLES 5 and 6 that after 20 days there was no cytotoxic activity (0 per cent survival and no prolongation of survival time of injected mice) in the sera of guinea pigs following inoculation of normal tissues. The

TABLE 5
CYTOTOXIC EFFECTS OF GUINEA PIG SERA FOLLOWING SUBCUTANEOUS
INJECTIONS OF NORMAL MOUSE TISSUE

Diluents used to prepare 1:1600 tumor-cell suspensions	Mice receiving intracerebral injections of tumor-cell suspensions	
	*	†
Pooled serum from animals injected with liver	15/15	(14.6)
Pooled serum from animals injected with spleen	15/15	(12.3)
Controls: pooled serum from animals injected with viable tumor	0/15	—
Controls: normal guinea pig serum	15/15	(14.0)

* Mice dying with growing tumors.

† Average time of survival, in days, of mice dying with tumor.

TABLE 6
CYTOTOXIC EFFECTS OF GUINEA PIG SERA FOLLOWING EYE INJECTIONS
OF NORMAL MOUSE TISSUES

Diluents used to prepare 1:1600 tumor-cell suspensions	Mice receiving intracerebral injections of tumor-cell suspensions	
	*	†
Pooled serum from animals injected with liver	10/10	15.4
Pooled serum from animals injected with spleen	10/10	16.9
Controls: pooled serum from animals injected with viable tumor	0/10	—
Controls: normal guinea pig serum	10/10	16.7

* Mice dying with growing tumors.

† Average time of survival, in days, of mice dying with tumor.

TABLE 7
CYTOTOXIC EFFECTS OF GUINEA PIG ANTISERA FOLLOWING
MULTIPLE INJECTIONS OF MOUSE TISSUES

Diluents used to prepare 1:1600 tumor-cell suspensions	Mice receiving intracerebral injections of tumor-cell suspensions	
	*	†
Pooled serum from animals receiving injections of killed S37 cells	0/15	—
Pooled serum from animals receiving injections of viable S37 cells	0/15	—
Pooled serum from animals receiving injections of killed liver cells	15/15	16.0
Pooled serum from animals receiving injections of fresh liver cells	0/15	—
Controls: normal guinea pig serum	15/15	16.0

* Mice dying with growing tumors.

† Average survival time, in days, of animals dying with tumor.

sera from guinea pigs that had received subcutaneous and intraocular injections of viable tumor were strongly cytotoxic.

(5) A fifth experiment was designed to study the cytotoxic effects of sera from guinea pigs that had received *multiple* injections of mouse tissues.

Four groups of 10 guinea pigs each received intraperitoneal injections of 1 ml. of 1:10 suspensions of the fresh liver, killed liver, and viable or nonviable tumor cells. The animals were injected 6 times at 3-day intervals, and were bled on the 20th day.

The data in TABLE 7 show that the sera from guinea pigs that had received multiple injections of viable and nonviable tumor cells and fresh liver, neutralized tumor-cell suspensions. There was no evidence of cytotoxicity in the serum of guinea pigs that had received multiple injections of killed mouse liver cell suspension.

Discussion

Cytotoxic activity against cells of mouse Sarcoma 37 appeared earliest in the sera of guinea pigs inoculated *subcutaneously* with mouse Sarcoma 37. The ultimate cytotoxic titers, however, were higher in animals that had received inoculations in the brain or in the eye. This finding parallels the earlier observation that inhibition of tumor growth is weaker after a preceding subcutaneous inoculation than after intracranial or intraocular inoculation.

Single inoculations of killed tumor tissue (subcutaneous or intraocular) failed to elicit detectable cytotoxins. *Multiple* (intraperitoneal) inoculations did elicit cytotoxins. Single inoculations of viable normal mouse tissue had an effect similar to that of killed tumor tissue. Cytotoxins were not found, but were detected after multiple inoculations. Multiple inoculations of killed normal tissue failed to elicit cytotoxins. It is conceivable that additional inoculation of killed normal tissues will give rise to detectable cytotoxins.

Summary

(1) We have described a sensitive method by which cytotoxins to mouse tumor cells are readily detected in the serum of guinea pigs inoculated with these tumors.

(2) Inoculation of viable mouse tumor to the eye or the brain of guinea pigs gave rise to a higher cytotoxic titer than did subcutaneous inoculation. Cytotoxins developed more rapidly, however, after subcutaneous inoculation.

(3) A single inoculation of killed tumor tissue or of viable normal mouse tissue did not give rise to detectable cytotoxins. Cytotoxins were found after multiple inoculations of either. Multiple inoculation of killed normal mouse tissue did not elicit detectable cytotoxins.

References

1. RAMBO, O. N., JR., R. FUSON, M. HATTORI & E. J. EICHWALD. 1954. Immune phenomena elicited by transplanted tumors. I. The participation of the eye and the brain. *Cancer Research*. **14**: 169-172.
2. EICHWALD, E. J. & H. Y. CILANG. 1951. The limitations of the anterior chamber in tumor transplantation using a serial sacrifice method. *Am. J. Pathol.* **27**: 740.
3. WERDER, A. A., A. KIRSCHBAUM, E. C. McDOWELL & J. T. SYVERTON. 1952. The in

- activation *in vitro* of transplantable myeloid and lymphoid mouse leukemic cells by antibodies produced in a foreign host species. *Cancer Research*. **12**: 886-889.
4. IMAGAWA, D. T., J. T. SYVERTON & J. J. BITTNER. 1951. The cytotoxic effect *in vitro* of antiserum upon heterologous mouse mammary cancer cells. *Cancer Research*. **11**: 259.
 5. GREEN, R. G. 1946. Cytotoxic properties of mouse cancer antiserum. *Proc. Soc. Exptl. Biol. Med.* **61**: 113-114.
 6. EICHWALD, E. J., C. J. GOODMAN & H. Y. CHANG. 1951. Tumor transplantation to the subdural space of heterologous hosts. *Proc. Soc. Exptl. Biol. Med.* **78**: 72-74.

Discussion of the Paper

GLENN H. ALGIRE (*National Cancer Institute, Bethesda, Md.*): O. N. Rambo, Jr., has presented a very interesting paper on a new method that may be used, not only to demonstrate cytotoxins to heterografts, but also to quantitate the immune response. Undoubtedly, readers have noted the similarity of this combined *in vitro-in vivo* approach to the methods described earlier in this monograph by Billingham and by Amos, for the demonstration of (to borrow Amos' term) "cytostatic" substances in the serum of mice that had been hyperimmunized to homografts between different mouse strains.

It is of interest that "cytostatic" substances have been found to homograft *only* by this combined *in vitro-in vivo* method. It is well known, however, that body fluids having a direct, injurious action on heterografts may be demonstrated by the methods of tissue culture (Harris, 1943). As Harris reminds us, earlier workers in cancer research (Lambert and Hanes, 1911; Lumsden *et al.*, 1934) were interested in cytotoxins as a possible antiserum against tumor cells, rather than in their significance as a factor in the transplantation of tissues. Subsequent work has not, as yet, substantiated the hopes of these workers.

I shall borrow one paragraph from the paper by Weaver, Prehn, and myself included later in this monograph, to indicate that cytotoxins to heterografts may be demonstrated in the living animal through the use of diffusion chambers. Diffusion chambers provide a method for maintaining grafted cells in good condition in the living animals, even though the graft is separated from cells of the host by a porous filter. Using mice that had been immunized by a single subcutaneous injection of human cells of the HeLa strain, one notes an accelerated destruction of HeLa cells in such mice, as compared with the nonimmune control animals (Algire *et al.*, 1956).

References

- HARRIS, M. 1943. The role of humoral antagonism in heteroplastic transplantation in mammals. *J. Exptl. Zool.* **93**: 131.
- LAMBERT, R. A. & F. M. HANES. 1911. The cultivation of tissues *in vitro* as a method for the study of cytotoxins. *J. Exptl. Med.* **14**: 453.
- LUMSDEN, T., T. F. MACRAE & E. SKIPPER. 1934. The direct demonstration of anti-cancer bodies in the serum of animals immune to a homologous tumor. *J. Pathol. Bacteriol.* **39**: 595.
- ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1957. Studies on tissue homotransplantation in mice, using diffusion-chamber methods. *Ann. N. Y. Acad. Sci.* **64**(5): 1009.

RICHARD B. STARK (*St. Luke's Hospital, New York, N. Y.*): I congratulate Rambo, Fuson, and Eichwald for their most interesting paper, and should like

to mention briefly a pilot experiment being undertaken at St. Luke's Hospital, New York, N. Y., by William Pyle, Janet Tait, and myself. This work is designed to explore the "dosage" phenomenon as it relates to the immune hypothesis of failure of homotransplants.

Using rabbits of divergent strains, macrografts of skin were transplanted between these animals so as to introduce a large dose of skin antigen. In some instances these grafts occupied the major portion of the dorsum of the animal. Running electrophoretic studies upon the 1st, 9th, 16th, 21st, and 30th days, we were surprised to find no quantitative changes in the spectra of serum proteins, including that of gamma globulin. We intend to increase the amount of skin antigen transplanted and to follow these studies with a series of successive grafts for further information concerning "later-set" transplants.

CHEMICAL STUDIES ON THE ENHANCING FACTOR*

By Andrew A. Kandutsch

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The term "enhancing factor" has been used to describe the substance(s) that cause(s) the breakdown of resistance to tumor homografts in host mice that have been pretreated with killed donor tissue. Previous attempts to isolate and identify this factor, or the presumably related XYZ or accelerant factors, have resulted in the preparation of active supernatant,¹ mitochondrial, and microsomal fractions^{2, 3} and, more recently, in a nucleoprotein prepared from isolated nuclei.⁴

The experiments to be described represent attempts to concentrate the enhancing factor and to obtain some clue to its identity by determining the stability of the substance, as measured by the retention of activity, when it is exposed to the influence of certain agents with more or less specific effects.

The system used in the present experiments to detect enhancing activity utilized mice of strain B10.D2 (a C57BL 10 subline) as hosts, and Sarcoma I, of strain A origin, as the source of the factor and as the homograft. Three intraperitoneal injections, 4 days apart, of killed tumor material were used to pretreat the hosts, and dosage was based on Kjeldahl nitrogen. The tumor was transplanted approximately 10 days after the last injection. Enhancing activity was indicated by the progressive growth of the tumor, to the death of the mouse. Only very rarely does Sarcoma I grow in untreated mice of this strain. This method of assay is lengthy and only crudely quantitative.

Activities of Tumor Fractions

Four main fractions, differing widely in chemical composition and in physical properties, were obtained by the procedures outlined in TABLE 1. A tumor homogenate was first divided into supernate and sediment after centrifuging for 30 minutes at $4500 \times g$. The supernatant material was further divided into substances precipitated by the addition of acid to pH 5.4 (fraction 1) and substances still in suspension or solution at this pH (fraction 2). The sediment from the initial separation was washed thoroughly at a low centrifugal speed and divided into 2 fractions by extraction with 1 M NaCl. The material soluble in 1 M NaCl was precipitated by diluting with water until the final salt concentration was 0.14 M NaCl. Solution and reprecipitation of this fraction was repeated twice (fraction 3). The material remaining after extraction with 1 M NaCl was washed several times with 1 M NaCl and with distilled water (fraction 4). All fractions were freeze-dried. The procedure used to obtain fraction 2 is similar to the first step commonly used to separate the ribonucleoprotein (RNAP) of tissues;⁵ 17 per cent of the lipid-free dry weight of this fraction was ribonucleic acid (RNA). Fraction 3 was obtained by a

* This investigation was supported by Research Grant C-1329 from the National Cancer Institute of the National Institutes of Health, United States Public Health Service, Bethesda, Md., and by a grant in-aid from the American Cancer Society, New York, N. Y., upon recommendation of the Committee on Growth of the National Research Council, Washington, D. C.

Part of this data will be published elsewhere in greater detail.

TABLE 1
TUMOR FRACTIONATION SCHEME
Tumor homogenized in saline
centrifuge 30 min. 4500 g

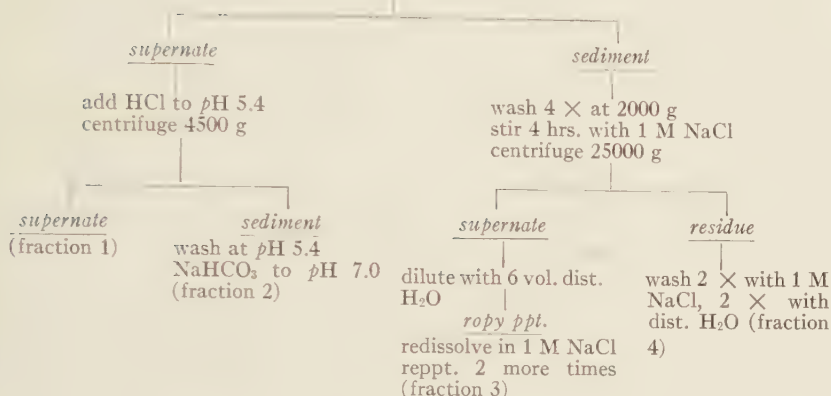


TABLE 2
ANALYSIS AND ACTIVITY OF TUMOR FRACTIONS*

Fraction no.	Description	mg. DNA mg. N†	mg. RNA mg. N†	mg. hexos- amine mg. N†	mg. lipid‡ mg. N†	Per cent of mice dying§
1	Supernatant pH 5.4	0.0	0.241	0.047	2.10	57
2	Ppt. pH 5.4 (RNAP)	0.013	1.023	0.022	3.44	67
3	Sol. 1 M NaCl (DNAP)	1.89	0.297	0.028	10.45	70
	Sol. 1 M NaCl (DNAP)	2.76	0.060	0.007	2.33	
4	Low speed sediment insol. in 1 M NaCl (residue)	0.143	0.102	0.034	2.11	83

* For methods used to determine DNA, RNA, and hexosamine see Dische,¹² Ceriotti,¹³ and Boas.¹⁴

† Kjeldahl nitrogen determined on lipid-free dry weight.

‡ Extracted with alcohol, followed by ether, in a soxhlet apparatus.

§ Three dose levels, 1.0, 0.3, and 0.03 mg. N per mouse, were administered to groups of 10 mice. Percentages were calculated from the total number dying out of 30.

procedure used for the isolation of desoxyribonucleoprotein (DNAP).⁵ Preparations obtained by this method were highly viscous in 1 M NaCl or distilled water, and DNA composed from 27 to 37 per cent of the lipid-free dry weight.

While the supernatant at pH 5.4 appeared to be less active than the other three fractions, enhancing activity did not appear to be particularly concentrated in any of these fractions (TABLE 2). Changes in activity less than fivefold, however, would probably not be detected with the test used. The experiment does serve several useful purposes. It indicates that DNAP was not required for activity (fractions 1, 2), and that activity was not proportional to the concentration of RNAP (compare fraction 2 with fractions 3 and 4). The second row of values under fraction 3 refers to an earlier preparation that contained only a trace of RNA, yet was highly active. Evidence that the RNAP does not possess enhancing activity was obtained by resuspending and

TABLE 3
EFFECTS OF EXTRACTION WITH ORGANIC SOLVENTS ON THE
ACTIVITY OF FREEZE-DRIED SARCOMA I*

Solvent	Temp. °C.	No. dying†
80% alcohol.....	50	0
Acetone.....	50	8
3:1 alcohol:ether.....	20	8
3:1 alcohol:ether.....	50	7
3:1 alcohol:ether.....	65 (reflux)	4
Saline.....	50	8
Saline.....	20	8
Uninjected.....		0

* Fifty mg. of solvent was added to 2 gm. of freeze-dried tumor. The mixture was heated to temperature for 2 hours, then allowed to stand overnight at room temperature. The insoluble residue was filtered off, washed with the proper solvent, dried, and injected at a level of 40 mg. dry weight per mouse.

† Ten mice per group.

reprecipitating the original precipitate obtained at pH 5.4. After 1 reprecipitation, considerable activity was lost; after 2 reprecipitations, the fraction was totally inactive.

The presence of hexosamine in all fractions was considered presumptive evidence for the presence of complex polysaccharides or mucoproteins, although hexosamine may be present as part of smaller molecules in the supernatant fraction (fraction 1). It is probable that some polysaccharide contaminates all or most nucleoprotein preparations, while part of the hexosamine in the residue fraction (fraction 4) may be accounted for by the presence of most of the connective tissue of the tumor.

Lipid was also present in all fractions. In agreement with Snell,² however, freeze-dried tumor retained a considerable degree of activity after the extraction of lipids with organic solvents (TABLE 3). Twenty-four per cent of the dry weight of freeze-dried tumor was extracted by a 3:1 alcohol:ether mixture, while smaller percentages were extracted by alcohol and acetone. Eighty per cent alcohol caused some decrease in activity at room temperature and complete loss of activity at 50° C. If the loss of activity resulting from extraction with 80 per cent alcohol is attributed to the denaturation of a protein, the explanation for the stability of the protein in the presence of the other organic solvents may be the relative dryness both of the active material and of the solvents. The lipid materials obtained in the extracts were tested for enhancing activity. Although, in some cases, tumors inoculated into mice pretreated with these lipids grew to a larger size than those in untreated mice, all of them eventually regressed. The observed growth-accelerating effect may be similar to the tumor growth-accelerating effect of lipid extracts reported by Miroff *et al.*⁶

Effects of Oxidizing Agents

The activity of a Sarcoma I fraction was rapidly lost after suspension in a dilute solution of sodium periodate (TABLE 4). Treatment with potassium permanganate also resulted in the loss of activity, although permanganate

TABLE 4
EFFECTS OF OXIDIZING AGENTS ON THE ACTIVITY OF A SARCOMA I FRACTION*

Treatment of material injected			No. of mice dying†	
Oxidizing agent	Molar conc.	Time exposed	1.5 mg. N	3.0 mg. N
None‡			9	8
NaIO ₄	0.01	1 hr.	0	1
NaIO ₄	0.01	20 min.	0	0
NaIO ₄	0.001	1 hr.	3	8
NaIO ₄ inactivated with glucose§	0.01	1 hr.	10	10
KMnO ₄	0.01	1 hr.	2	
KMnO ₄	0.001	1 hr.	6	
H ₂ O ₂	0.01	1 hr.	10	
K ₂ Cr ₂ O ₇	0.01	1 hr.	8	

* Three hundred and sixty-four or 728 mg. of Sarcoma I sediment (2000 × g) suspended in 20 ml. of buffered saline (pH 7) containing the oxidizing agent. After the proper time interval, the suspension was centrifuged and the sediment washed with a solution containing 10 per cent glucose and 0.14 M NaCl. The washed sediment was resuspended in saline and injected.

† No. dying out of 10 mice per group; dose injected per mouse.

‡ Numbers of mice dying that received untreated material at levels of 0.5 and 0.05 mg. N injected per mouse were 9 and 0 respectively.

§ An excess of glucose was added to the solution of NaIO₄ before the tumor material was suspended in it.

appeared to be less effective than periodate at the same concentration. Treatment with sodium periodate, previously inactivated by the addition of glucose, or with hydrogen peroxide or potassium dichromate, had no effect on the activity of the tumor fraction.

The marked effect of sodium periodate, in contrast to the weaker effect or total ineffectiveness of the other strong oxidants, suggests that the sodium periodate ion may have acted in its relatively specific capacity to split carbon-carbon bonds where hydroxyl or hydroxyl and amino groups are adjacent. This hypothesis is particularly attractive since the presence of a polysaccharide was indicated by hexosamine analyses, and because of the supposed antigenic character of the enhancing factor.⁷ The periodate ion is known to inactivate the blood group and other polysaccharide-containing antigens, and there is a marked similarity between the results shown in TABLE 4 and those obtained by Hirst in a study on the carbohydrate-containing virus receptors of red blood cells.⁸ On the other hand, periodate also oxidizes the hydroxyamino acids and has a less well-understood inactivating effect on several biologically active proteins.^{9, 10, 11}

Effects of Certain Enzymes

The presence of a protein essential for activity was suggested by the lability of the factor to heat,^{1, 3} 80 per cent alcohol, and 90 per cent phenol (unpublished data). To test the effects of trypsin and of other enzymes, washed material sedimented from a tumor homogenate at low centrifugal speeds was incubated for 4 hours with the enzyme. The mixture of tumor and enzyme was then diluted to the proper concentration and injected. As shown in TABLE 5, both trypsin and hyaluronidase caused a decrease in activity under these conditions. When the hyaluronidase was removed from the tumor material by

TABLE 5

EFFECTS OF CERTAIN ENZYMES ON THE ACTIVITY OF A SARCOMA I FRACTION*

Enzyme	Per cent of mice dying†		
	1.5 mg. N	0.5 mg. N	0.5 mg. N (washed)‡
None	65	85	
Trypsin	10	5	
Hyaluronidase	25	35	90
Desoxyribonuclease	80	50	80

* Concentrations: 270-340 mg. tumor sediment in 25 ml. buffered saline containing 50 mg. crystalline trypsin, 50 mg. crystalline hyaluronidase, or 5 mg. crystalline desoxyribonuclease. MnCl_2 added to DNase incubation mixture at a level of 0.025 M.

† Total of 10 or 20 mice per group. Each animal received the dose indicated.

‡ After the incubation period the enzyme was removed by centrifuging at $25000 \times g$ and discarding the supernate.

centrifugation, however, after the incubation period, it appeared to have little effect on activity. Trypsin could not easily be removed in the same way, since this enzyme caused the tumor fraction to become so very viscous that it would not be completely sedimented even at $25000 \times g$. Experiments designed to determine the effects of injected trypsin and hyaluronidase are in progress.

Desoxyribonuclease did not decrease the activity of the tumor fraction; on the contrary, in one experiment it resulted in a definite increase in activity. Preliminary studies with ribonuclease and with lysozyme indicate that these enzymes do not affect enhancing activity.

Summary and Conclusions

These data show that enhancing activity was present in all tissue fractions similar to those previously reported to possess enhancing or accelerant activity and was also present in a residue fraction that remained insoluble after thorough extraction with 0.14 M NaCl, 1 M NaCl, and distilled water. The activity did not appear to be specifically concentrated in any one fraction, and it could not be associated with RNAP, DNAP, or lipid.

While the data cannot be considered definitive, they suggest that at least 2 components, one a protein and the other a carbohydrate, may be essential for activity. A protein is indicated by the lability of the factor to heat, 90 per cent phenol, 80 per cent alcohol, and to digestion with trypsin. The presence of a carbohydrate component is suggested by the marked inactivating effect of sodium periodate as contrasted with the effects of other strong oxidizing agents. The presence of hexosamine in all active fractions and the generally assumed immunological nature of the enhancing phenomenon suggest that the 2 components may be present in the form of a mucoprotein.

Acknowledgments

Much of the work reported here was done in collaboration with Ursula Wenck. The valuable advice of George D. Snell and Nathan Kaliss is gratefully acknowledged.

References

1. DAY, E. C., N. KALISS, A. I. ARONSON, B. F. BRYANT, D. FRIENDLY, F. C. GABRIELSON & P. M. SMITH. 1954. *J. Natl. Cancer Inst.* **15**: 145-159.
2. SNELL, G. D. 1952. *J. Natl. Cancer Inst.* **13**: 719-729.
3. SHEAR, H. T., J. T. SYVERTON & J. J. BITTNER. 1954. *Cancer Research*. **14**: 175-182.
4. HORN, E. C. 1955. *Cancer Research*. **15**: 663-670.
5. MIRSKY, A. E. & A. W. POLLISTER. 1946. *J. Gen. Physiol.* **30**: 117-147.
6. MIROFF, G., C. MARTINEZ & J. J. BITTNER. 1955. *Cancer Research*. **15**: 437-441.
7. SNELL, G. D. 1954. *J. Natl. Cancer Inst.* **15**: 665-675.
8. HIRST, G. K. 1948. *J. Exptl. Med.* **87**: 301-314.
9. GOEBEL, W. F., P. K. OLITSKY & A. C. SAENZ. 1948. *J. Exptl. Med.* **87**: 445-455.
10. GOEBEL, W. F. & G. E. PERLMANN. 1949. *J. Exptl. Med.* **89**: 479-489.
11. JANSEN, E. F., A. L. CURL & A. K. BALLS. 1951. *J. Biol. Chem.* **189**: 671-682.
12. DISCHE, Z. 1955. Color reactions of nucleic acid components. *In The Nucleic Acids*. **1**: 287-290. E. Chargaff & J. W. Davidson, Eds. Acad. Press. New York, N. Y.
13. CERIOTTI, G. 1955. *J. Biol. Chem.* **214**: 59-70.
14. BOAS, N. F. 1953. *J. Biol. Chem.* **204**: 553-563.

Discussion of the Paper

HENRY J. WINN (Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.): Immunologists have long been aware of the difficulties involved in trying to isolate and purify tissue antigens. In the work reported by Andrew A. Kandutsch these difficulties have become exaggerated because of the nature of the assay system that must be used. This procedure is quite laborious and requires considerable expenditure of time and animals. In addition, it should be pointed out that the procedure tests not only the specificity of the tissue preparations, but also the complete antigenicity, that is, the ability to engender the formation of specific antibody. Conclusions drawn with regard to the chemical nature of the enhancing substance should be considered in light of this fact. I should like to suggest that the suspected protein portion may be functioning as a carrier to insure proper contact between the enhancing substance and the antibody-forming cells of the host, and that the specificity resides in the nonprotein (polysaccharide) portion of the complex. It is, of course, quite possible that the combining sites of antibodies for the enhancing substance are directed partially toward the protein moiety, as is the case in some antibody for the artificially conjugated azo proteins. I think, however, that the differences between H-2 genotypes may involve only structural differences in nonprotein cell substances.

For similar reasons one cannot be certain that the organic solvent extracts did not contain some hapten that would, of course, require a schlepper to become manifest in enhancing experiments. However, most of the extracted residues showed significant enhancing activity; if extraction was complete, this could mean that the extracts were completely inactive.

With regard to the possibility of using other assay systems, I should like to state that recently I have had the opportunity to undertake some joint work with Kandutsch and that we are trying to devise a suitable serologic method of assaying tissue fractions for the H-2 antigens. The technique of hemagglutination inhibition would appear, on first consideration, to be ideal, since these antigens are known to be present on mouse red cells. Unfortunately, mouse

isohemagglutinins are quite unstable and are often of the incomplete or non-agglutinating type. The animals used in these enhancing studies differ from the tumor-donor strain at histocompatibility loci other than H-2, but all of the evidence so far adduced indicates the sole importance of the latter locus in enhancing studies with this particular host-donor-tumor combination. Moreover, the use of isogenic-resistant mouse strains can obviate the necessity of making allowances for other loci, and studies with these isogenic-resistant lines substantiate the preeminent role of the H-2 antigens.

Part IV. Lymphocytes, Lymph Nodes, and Homotransplantation

STUDIES ON TISSUE HOMOTRANSPLANTATION IN MICE, USING DIFFUSION-CHAMBER METHODS

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It is generally accepted, from the work of Medawar¹ and of others, that the reaction of the host against homografts is based on a mechanism of actively acquired immunity. It has long been debated, however, whether destruction of homografts is caused by cells, by antibodies or cytotoxins in the serum, or by both. This paper is a brief account of our studies on this problem.

In order to facilitate observations on the possible role of these factors, we developed methods by means of which implanted cells, although isolated from contact with cells of the host by a porous filter, could survive and proliferate within living mice.^{2, 3}

The earliest paper we have found on a method intended for this purpose was that by Rezzesi⁴ in 1932. In this procedure, as used by Bisceglie,⁵ a collodion bag containing the graft was tied at the end and placed within the peritoneal cavity. It was reported that the Ehrlich carcinoma of mice survived for 12 days in a guinea pig under these conditions.

The recent availability of filters of cellulose nitrate or of cellulose esters of graded porosity from several sources*, enabled us to construct two types of diffusion chambers. The average porosity of the Millipore filters used in our work, except as indicated, was stated by the manufacturers to be 0.45μ , small enough to prevent the passage of cells but presumably large enough to let through proteins.

For observation of living cells, the filters were adapted for use with a transparent chamber inserted under the skin.² The filters are relatively opaque, but a portion of the filter, 3 mm. in diameter, was made transparent so as to allow observation of the living cells both within the chamber and on the tissues beneath the chamber.

A second type of chamber intended for histologic study was placed intraperitoneally and removed for fixation after varying intervals of time.

Inbred strains of mice were used in these experiments. I shall refer to grafts within the same inbred strain as "isologous." These, as is well known, usually survive. Grafts between mice of different inbred strains will be referred to as homografts. These, of course, are usually rejected by the host.

It was known from the work of Merwin and Hill⁶ that small homografts, placed so as not to become vascularized, will survive indefinitely in the subcutaneous site because they do not initiate immunity in the host. It seemed likely, therefore, that homografts would survive in diffusion chambers in non-immune hosts, and this proved to be the case.

* Millipore Filter Corp., Watertown, Mass.; Carl Schleicher and Shuell Co., Keene, N. H.; F. M. Himmelweit, the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, Paddington, London, England.

Since it was known also that nonvascularized homografts in the subcutaneous site were destroyed in an immune host,⁶ the next step was to learn whether homografts would survive in diffusion chambers in immunized animals.

Using the diffusion chamber as adapted for observation of living cells, a homograft of Harderian gland was placed within the chamber and, as a control, another graft was placed upon the tissues of mice that had been immunized by a single inoculation of spleen from the foreign strain 2 weeks earlier. It was known from previous work⁶ that such grafts in contact with host tissues were destroyed within 3 to 6 days.

The implants *in contact* with host cells and tissues were destroyed within 3 days, while grafts separated from host cells by a filter survived for the duration of the experiments (11 to 21 days).² In this manner, the filter protected the homograft from destruction in immune hosts. Under these conditions, grafts of both normal and tumor tissue have survived in experiments extending as long as 4 to 6 months.

While cells from immunized hosts were excluded, possibly other things of importance were also excluded. Experiments were next carried out to gain more information on the possible role of cells and on the role of the cell types involved in the destruction of homografts, as well as to show that *some* antibodies or cytotoxins, if present, could pass through the filters.

In these experiments⁷ mice were immunized approximately 2 weeks before implantation of the chambers by a *single* inoculation of a spleen fragment by trocar. Grafts of mouse-embryonic lung rudiments, skin epithelium, or mammary adenocarcinoma, referred to hereafter as "target" tissues, were placed within the diffusion chambers, which in turn were placed within the peritoneal cavity. Histologic preparations of the diffusion chambers and contents were made approximately 6 days after their implantation into the mouse. In all cases, using filters that did not allow the passage of host cells, the target cells survived and continued to proliferate.

In a second series of experiments, filters of greater porosity were used (Type AA millipore*) so that leukocytes and macrophages of the host could enter the chamber and come in contact with the target tissue. Destruction of the target cells was seen if the cells entered the chambers from immune hosts, but not if cells entered from nonimmune homologous hosts.

Not all of the types of cells that entered the chambers from the host appeared to participate in the destruction of the grafts, nor was destruction accomplished by phagocytosis. Histologic evidence suggested that the cells that destroyed the homografts were lymphocytes.

The question arose as to whether the filters excluded not only cells but also antibodies that might be present in the extracellular fluid. This seemed unlikely, since *in vitro* studies showed that the filters allowed the quantitative passage of equine gamma globulin and of diphtheria antitoxin. Further *in vitro* studies, however, were undertaken using heterografts, which are known to elicit humoral cytotoxic substances that can be demonstrated *in vitro* or by tissue-culture methods.⁸ Diffusion chambers containing lung rudiments or epidermal cells from mice were implanted into cell-impermeable chambers that

* See footnote, previous page.

were then placed in rats previously immunized to these target cells. The target cells were killed or injured after 2 days in the immunized heterologous hosts, but survived without signs of injury for at least a week in normal rats.

It seems reasonable to assume that destruction of the targets was caused by cytotoxins to these heterografts. One would expect that cytotoxins to homografts, if present, would also be capable of passing through these membranes.

In more recent preliminary experiments (unpublished) we considered the possibility that the pores, even though initially permeable to humoral cytotoxins, might become blocked after a short time in the peritoneal cavity of the mouse. Heterografts (HeLa strain of human cells) were placed in cell-impermeable chambers.

After 5 days in nonimmune mice, these chambers were transferred to mice previously immunized against HeLa cells. Cells in these chambers showed extensive destruction after 7 days in the immunized hosts, as compared with the controls placed in nonimmune second hosts. These results indicate that cytotoxins to these heterografts can pass through the pores of chambers that had been in the peritoneal cavity for several days.

Further experiments were undertaken* to determine whether hemolysins in mice immunized by sheep red blood cells entered the chambers *in vivo*. Determinations of hemolysin titers were made both from sera and from filtrates obtained *in vivo*, using type HA millipore filters. The filtrates were obtained by using a modified diffusion chamber in which the filters were separated by a space of 5 mm. Sheep red blood cells were injected into mice the same day that diffusion chambers were introduced, following procedures described elsewhere⁹ for immunization and estimation of hemolysin titers. Titers of pooled serum from 5 mice were 1:744, and of filtrate 1:139, respectively. It is apparent that mouse antish sheep hemolysins pass through these filters *in vivo*, although in lower concentration than in the serum. Further work will be needed to determine hemolysin titers in the individual animals, and in ascitic fluid as compared to serum.

It has also been important to know more about the composition of fluids passing through these filters in the living animal. Analyses for total protein or nitrogen were made on filtrates obtained from chambers constructed using type HA millipore filters†. The results shown in TABLE 1 are in agreement with published data on protein content of ascitic fluid of mice.¹⁰ Preliminary paper electrophoresis determinations of protein in filtrates from these chambers showed no significant qualitative differences from that of serum, nor did it reveal any evidence of selective filtration.

In a final series of experiments⁷ we considered the possibility that a humoral factor from the circulating blood of the immune host might also be of importance in homograft destruction. Experiments were undertaken with the object of testing cells alone. Spleen from homologous immunized mice, as well as from nonimmunized mice, was used as a source of living cells.

The cells, washed to remove antibodies possibly present in the tissue fluids,

* We acknowledge with thanks the cooperation of Falconer Smith, National Cancer Institute, Bethesda, Md., in making these determinations.

† We are indebted to Benton B. Westfall for determinations of total protein; to Mrs. Mary M. Wyckoff for paper electrophoresis analyses; and to R. J. Koegel and his staff, National Cancer Institute, Bethesda, Md., for nitrogen determinations.

TABLE 1
DETERMINATIONS OF NITROGEN AND PROTEIN IN SERUM AND IN FILTRATES
FROM INTRAPERITONEAL DIFFUSION CHAMBERS

Filtrate	Serum
3.92 mg. N/ml.	6.74 mg. N/ml.
5.11 mg. N/ml.	6.59 mg. N/ml.
3.9% protein	
4% protein	
4.4% protein	

were placed in cell-impermeable chambers together with the target tissues. The chambers were then placed in hosts of the same strain from which the target cells were taken. Extensive destruction of the target cells occurred only if the spleen cells came from immunized hosts. So far as one can decide from histologic observation, it appears that lymphocytes are involved in the destruction of the homografts, that intimate contact is required, and that both lymphocytes and target cells are destroyed when this occurs.

Accordingly, everything necessary for destruction is present when a washed suspension of spleen cells is combined with target cells in diffusion chambers *in vivo*, and nothing is required from an immunized homologous host.

In conclusion, the results of these experiments are in agreement with the hypothesis that cytotoxins to homografts are transported by cells. Histologic evidence strongly suggests that lymphocytes are involved in this destruction, but stronger proof of this is needed.

References

1. MEDAWAR, P. B. 1954. General problems of immunity. *In* Preservation and Transplantation of Normal Tissues. Ciba Foundation Symposium. J. & A. Churchill, Ltd. London, England.
2. ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1954. Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in immunized mice. *J. Natl. Cancer Inst.* **15**: 493.
3. PREHN, R. T., G. H. ALGIRE & J. M. WEAVER. 1954. The diffusion chamber technique applied to the homograft resistance mechanism. *J. Natl. Cancer Inst.* **15**: 509.
4. REZZESI, F. D. 1932. Eine Methode zur Züchtung der Gewebe *in vivo*. *Arch. Exptl. Zellforsch. Gewebezücht.* **13**: 258-281.
5. BISCEGLIE, V. 1933. Über die antineoplastische Immunität. II. Mitteilung. Über die Wachstumsfähigkeit der heterologen Geschwülste in erwachsenen Tieren nach Einpflanzung in Kollodiumsäckchen. *Z. Krebsforsch.* **40**: 141-158.
6. MERWIN, R. M. & E. L. HILL. 1954. Fate of vascularized and nonvascularized subcutaneous homografts in mice. *J. Natl. Cancer Inst.* **14**: 819.
7. WEAVER, J. M., G. H. ALGIRE & R. T. PREHN. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. *J. Natl. Cancer Inst.* **15**: 1737.
8. HARRIS, M. 1943. The role of humoral antagonism in heteroplastic transplantation in mammals. *J. Exptl. Zool.* **93**: 131.
9. SMITH, F. & H. J. RUTH. 1955. Hemolysin production in irradiated mice given spleen or bone marrow homogenate. *Proc. Soc. Exptl. Biol. Med.* **90**: 187.
10. LEDOUX, L., & S. H. REVELL. 1955. Action of ribonuclease on neoplastic growth. I. Chemical aspects of normal tumour growth: the Landschütz ascites tumour. *Biochim. et Biophys. Acta.* **18**: 416.

Discussion of the Paper

GLENN H. ALGIRE: I see no fundamental contradiction between our results and the findings of those who have used combined *in vitro-in vivo* methods. Evidence for cytostatic or cytotoxic humoral substances to homografts have been obtained with these methods by Gorer,¹ and as represented at this conference by Billingham and by Amos.

Our failure to find evidence of such humoral substances to homografts in our completely *in vivo* experiments, as compared to their combined *in vitro-in vivo* procedures, may reflect only differences in the degree of immunity. In contrast to theirs, our animals were not hyperimmunized, yet in our experiments the immunity was strong enough to injure severely or to destroy any graft to which host cells could gain access.

Reference

1. GORER, P. A. 1955. The antibody response to skin homografts in mice. Ann. N. Y. Acad. Sci. **59**(3): 365.

CELLULAR AND HUMORAL FACTORS IN THE IMMUNITY TO SKIN HOMOGRAFTS: EXPERIMENTS WITH A POROUS MEMBRANE*

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The development of porous membranes that readily permit the passage of large molecules while preventing the passage of cells has provided a valuable tool for the study of immunity to homografts and heterografts.

Important results have already been reported by Algire and his colleagues (Algire, Weaver, and Prehn, 1954; Prehn, Weaver, and Algire, 1954; Weaver, Algire and Prehn, 1955; Prehn, Algire, and Weaver, 1955), who enclosed a variety of mouse tissues, including harderian gland, embryonic heart and lung, epidermal cell suspensions, and also neoplastic tissue, in "diffusion chambers" constructed from such membranes, and then inserted the chambers intraperitoneally or subcutaneously in normal mice and rats, and also in mice and rats immunized by a previous graft of orthodox type from another animal of the donor strain.

In the present experiments, a technique has been devised using porous membrane in conjunction with split-skin grafts. We believe that this procedure embodies several advantages. In the first place, skin homografts have been studied more extensively than homografts of any other normal tissue. Second, skin can be transplanted orthotopically, that is, to an anatomically natural site. Third, the membrane can be removed and the graft transferred to another site with virtually no disturbance of the structure of the graft.

Technique

The technique used in most of the experiments is illustrated in FIGURES 1 to 6. A split-skin graft is cut with a Padgett-type dermatome from the donor rat, as described by Woodruff and Simpson (1955). The graft is trimmed to a square 2×2 cm., placed raw surface upward on a sterile glass plate, and centered over a square of white paper (3×3 cm.) beneath the plate. A square of membrane (2.5×2.5 cm.) that has been sterilized by immersion in 70 per cent alcohol for half an hour and dried in an incubator at 37° C. is placed over the graft and made to adhere to it by gentle pressure, the graft being centered by reference to the square of paper under the glass plate. The membrane with the graft adhering to it is then inverted so that the outer surface of the graft is uppermost, and a square of lightly greased sterile cellophane is placed over the graft and membrane and made to adhere by gentle pressure with a glass roller. It is convenient to have the cellophane slightly larger than the piece of membrane; later, it can be trimmed to size with scissors.

A bed is prepared on the host by removing a square of skin 2.5×2.5 cm. down to the panniculus carnosus as described by Woodruff and Simpson (1955). The skin at the edges of the defect is undermined for a distance of 0.5 cm. or a

* This work was supported in part by a grant from the Medical Research Council of New Zealand, Wellington, New Zealand.

little more. The size of the defect is temporarily increased by traction on the skin, and the sandwich of membrane, graft, and cellophane is laid on the panniculus, to which it readily adheres. A piece ($2.5 \times 2.5 \times 0.3$ cm.) of plastic sponge is placed over the sandwich after the area has been lightly dusted with penicillin-lactose powder, and the skin is sutured across the corners. After a further dusting with penicillin-lactose powder a square of tulle gras is applied and the whole area is covered with 3 turns of open-weave bandage and two turns of plaster-of-Paris bandage. The animal's serial number is inscribed on the plaster with an indelible pencil (FIGURE 9).

As a rule, this technique gives excellent results but occasionally the cellophane becomes detached from the membrane and host cells gain access to the graft. To obviate this, the graft may alternatively be sandwiched between two squares of membrane that are sealed together along their edges with acetone by the method shown in FIGURES 7 and 8. A third method, that so far has been given only a preliminary trial, is similar to the first procedure, with the exception that the cellophane is cut to the size of the graft and both are covered with a larger square (2.5×2.5 cm.) of adhesive material. Such material must retain its adhesive property during sterilization and also while in the host animal. Commercial adhesive cellulose tape fulfills the first of these criteria, but not the second. Experiments with other materials are in progress and will be reported later.

Experiments and Results

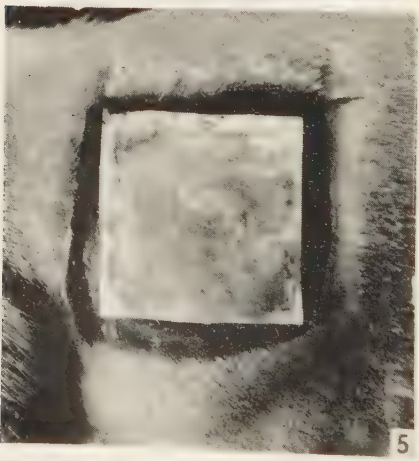
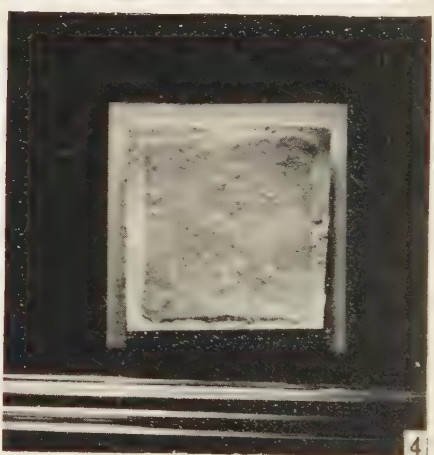
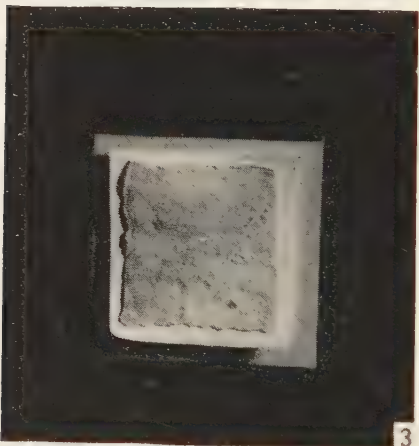
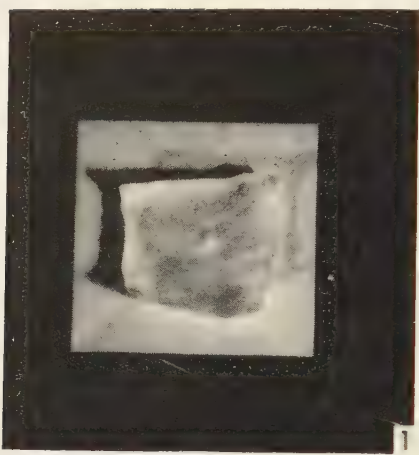
Throughout the investigation, black and white hooded rats, aged 4 months and weighing between 200 and 250 gm., were used as homograft donors, and Wistar-strain rats of the same age were used as recipients. The membrane used was millipore HA, 150μ thick*. When, as sometimes happened as a result of faulty technique, host cells gained access to grafts over membrane, the animal was excluded from the experiment.

Preliminary Observations

The first step was to check the period of survival of ordinary grafts, as previous observations (Woodruff and Simpson, 1955) had been made with slightly older rats. This was found to be 12 days (range 11 to 13 days) for first-set grafts, and 5 days for second-set grafts in animals that had received a first-set graft from the same donor 4 weeks previously.

It was next shown that autografts and homografts over membrane were still viable after 13 days. At the end of this period, histological examination usually showed that the surface epithelium had desquamated, but in the dermis there were viable connective tissue cells and also viable epithelial cells in the hair follicles (FIGURE 10). Autografts transferred to a new area without membrane and homografts replaced on the donor without membrane behaved similarly, and histological examination after 7 days showed complete regeneration of surface epithelium (FIGURE 11).

* Made by the Millipore Filter Corp., Watertown, Mass. Preliminary trials with AA membrane of the same thickness were unsatisfactory because the pores of the membrane became clogged with portions of disintegrating host leukocytes. It was learned subsequently that the same phenomenon had been observed by Algire *et al.* (1954).



TECHNIQUE OF GRAFTING SKIN OVER MILLIPORE MEMBRANE

FIGURE 1. Graft (2×2 cm.) placed raw surface upward on a sterile glass plate, and centered over a square of white paper (3×3 cm.) underneath the plate.

FIGURE 2. Square of membrane (2.5×2.5 cm.) placed over the graft and centered by reference to the square of paper beneath the glass plate.

FIGURE 3. Membrane with adherent graft inverted so that the outer surface of the graft is uppermost.

FIGURE 4. Square of lightly greased sterile cellophane placed over graft and membrane, and made to adhere by gentle pressure with a glass roller.

FIGURE 5. Sandwich of membrane, graft and cellophane placed on panniculus carnosus of host.

FIGURE 6. Square of sterile plastic sponge placed over the sandwich, and skin sutured across the corners.



7



8



9

TECHNIQUE OF GRAFTING SKIN OVER MILLIPORE (CONTINUED)

FIGURES 7 and 8. An alternative technique. The graft is placed between 2 squares of membrane and the whole assembly is held in the specially designed forceps (FIGURE 7) while the squares are sealed together along each edge by applying acetone with a fine brush.

FIGURE 9. The graft is in place. The area has been dusted with penicillin-lactose powder and covered with a square of tulle gras, 3 turns of open-weave bandage, and 2 turns of plaster-of-Paris bandage.



FIGURE 10. Skin homograft over millipore membrane after 13 days. $\times 150$. The surface epithelium has been lost but there is still viable epithelium in the hair follicles.

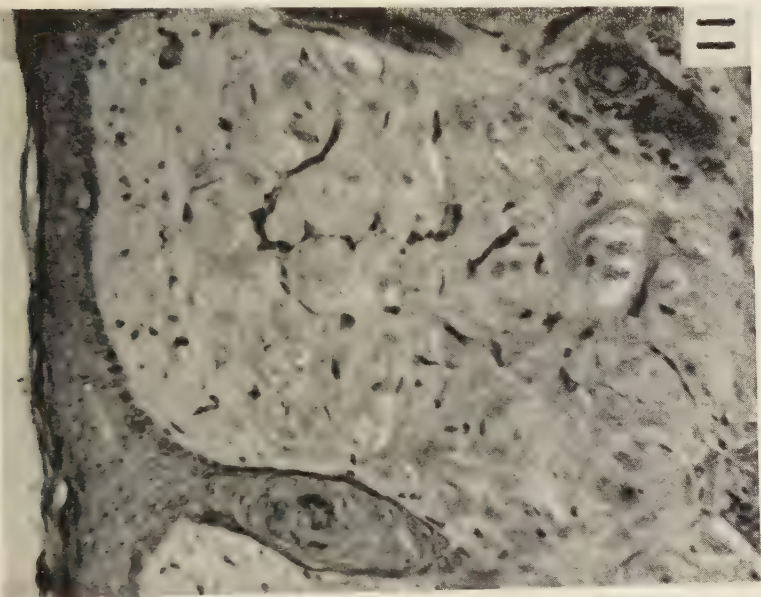
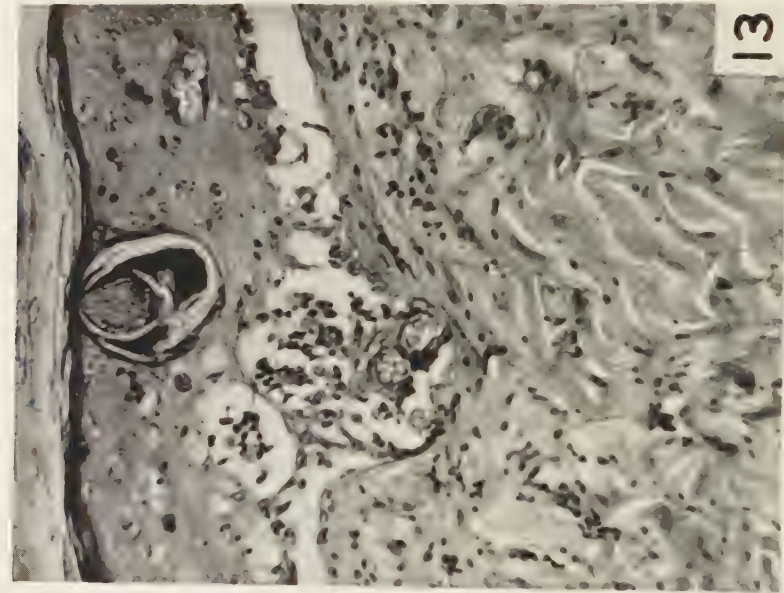
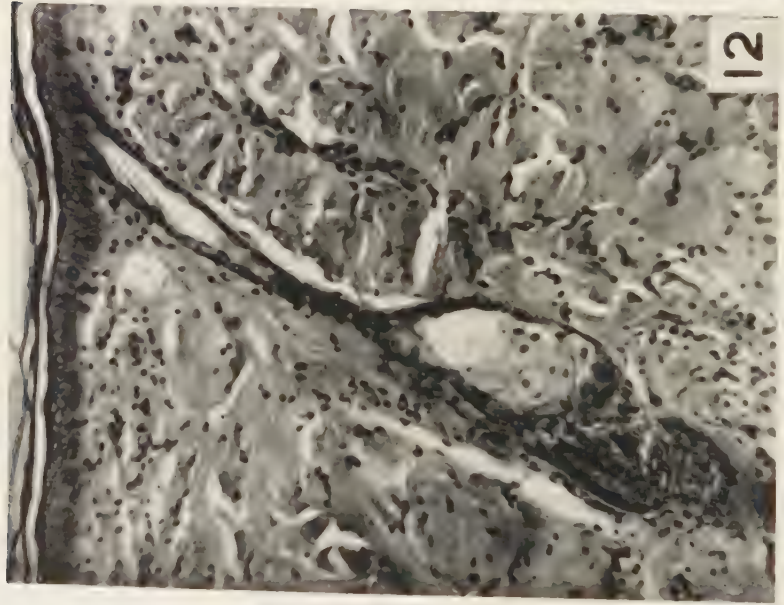


FIGURE 11. Part of the same graft as in FIGURE 10 after being replaced on the donor as an ordinary graft for 7 days, showing epithelial regeneration. $\times 150$.



FIGURES 12 and 13. Ordinary homograft after 8 days and 12 days, respectively, in an animal that had previously received a homograft over a month from the same donor. Breakdown is just complete in FIGURE 13. $\times 150$.

In the light of these preliminary observations, experiments were designed to determine: first, whether a homograft isolated by membrane from contact with host cells is effectively antigenic; and, second, whether the behavior of such a homograft is modified by previously immunizing the host with an ordinary graft from the same donor.

First Experiment

The host received a split-skin homograft over membrane, which was removed 13 days later. After a further 15 days, the host received an ordinary split-skin homograft to the opposite side of the chest from the same donor. The period of survival of this second graft (FIGURES 12 and 13) in 9 recipients, determined by serial biopsy performed 8, 12, and 16 days after grafting, ranged from 11 to 13 days, with a mean of 12 days.

Second Experiment

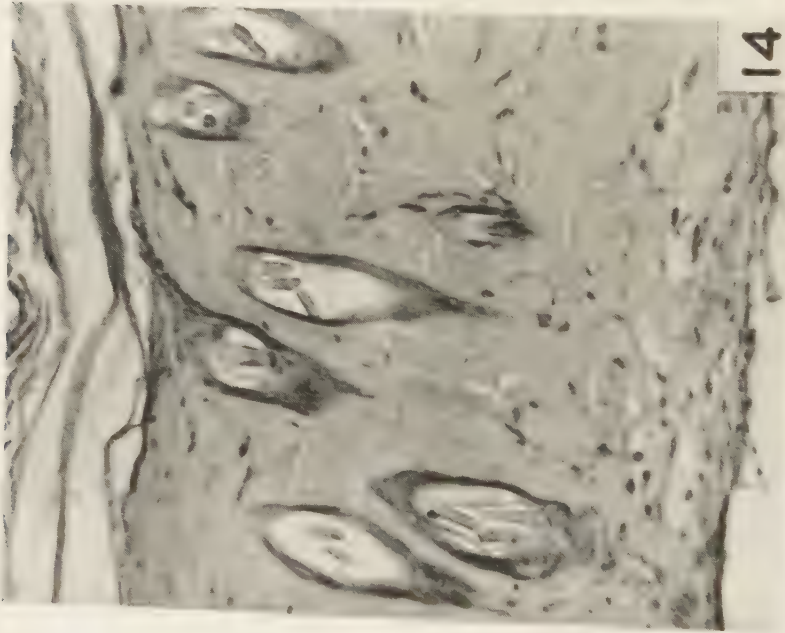
The host received a split-skin homograft over membrane. After 13 days a small piece of the graft was taken for section and the remainder was transferred to a fresh defect in the host, on the opposite side of the chest, without membrane. Biopsy was performed 8, 12, and 16 days after transfer. In addition, in some cases, a small piece of the graft was removed 5 days after transfer and replaced on the donor.

The period of survival of the grafts after transfer in 7 recipients ranged from 11 to 14 days, with a mean of 12 days. Grafts replaced on the donor 5 days after transfer to a new area in the host all survived. The findings in one pair of animals are illustrated in FIGURES 14 to 17.

Third Experiment

The host received an ordinary split-skin homograft as an immunizing graft. After 28 days the host received a second split-skin homograft from the same donor to the opposite side of the chest, this time over membrane. After 13 days the second graft was removed and divided into 3 portions. One of these was sectioned, one was replaced on the donor, and one was transferred without membrane to a new area in the host. The portion of graft replaced on the donor was removed and sectioned after a further 7 days. The portion transferred to a new area in the host was examined and biopsied daily, or in some cases every second day.

The grafts over membrane in 6 immunized recipients all proved to be viable after 13 days, as judged by histological examination before and 7 days after they were replaced on the corresponding donor. On transfer to a new area in the host, the grafts never became firmly attached and an intense inflammatory reaction, characterized by accumulation of polymorphonuclear leukocytes, developed in the graft bed. The extent to which host cells penetrated into the transferred grafts varied greatly in different grafts and also in different parts of the same graft. Polymorphs were sometimes found in grafts as early as 2 days after transfer, and wherever they occurred the epithelial cells in the vicinity appeared to be dead. On the other hand, in parts of the grafts not

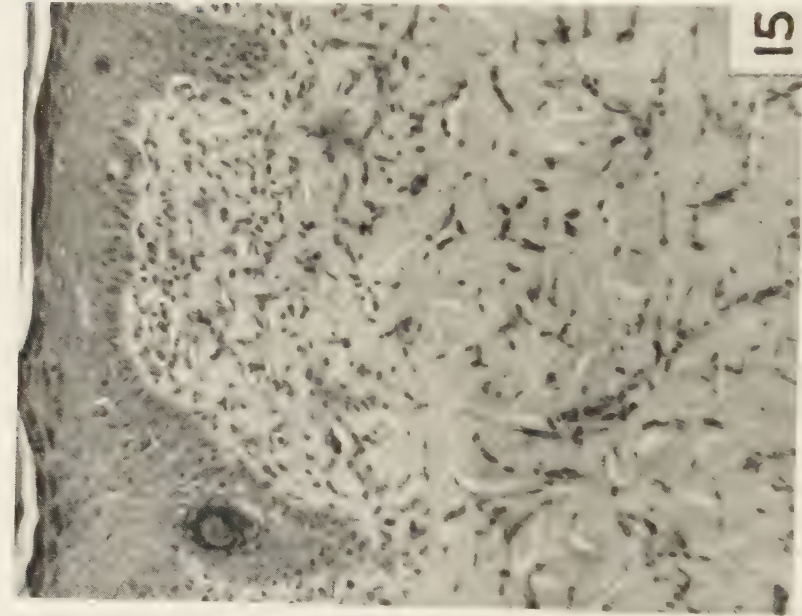


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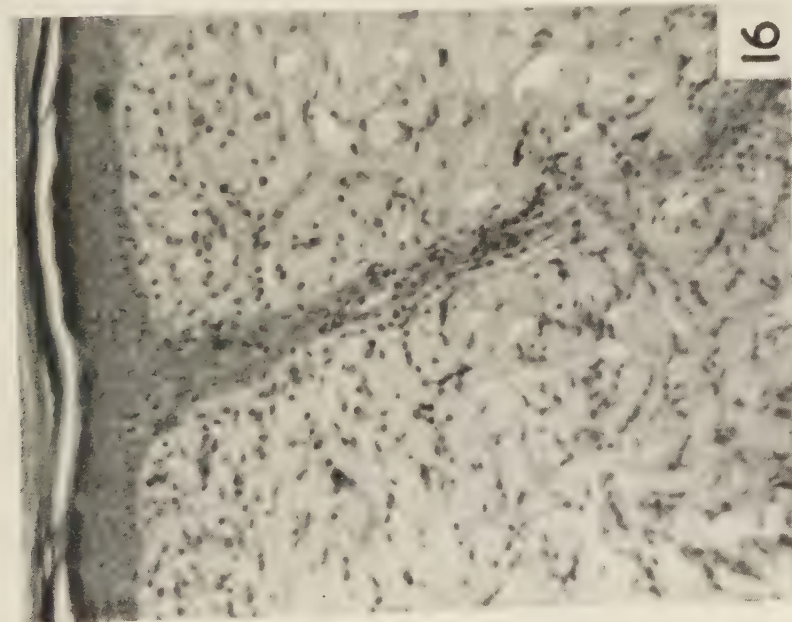
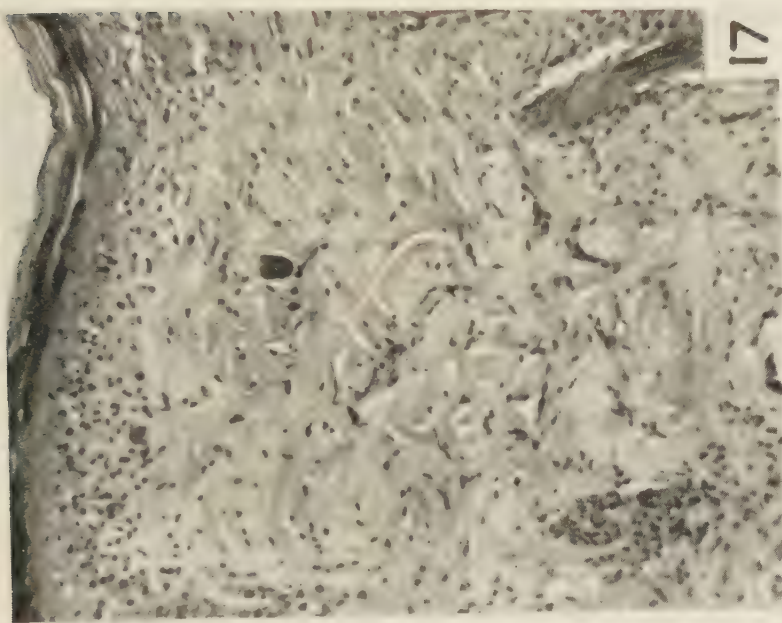
FIGURE 14. Homograft over millipore after 13 days. $\times 150$.

PLATE 1

Part of the homograft after transfer to a new area on the host for 5 days and then back to the donor for a further 7 days. $\times 150$.



15



FIGURES 16 and 17. Part of the same graft 8 days and 12 days after transfer as an ordinary graft to a new area of host. $\times 150$.

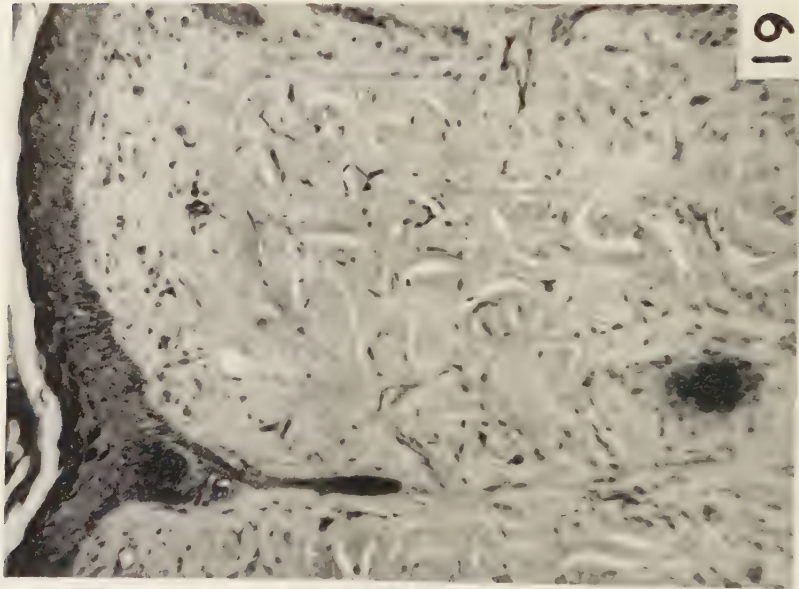
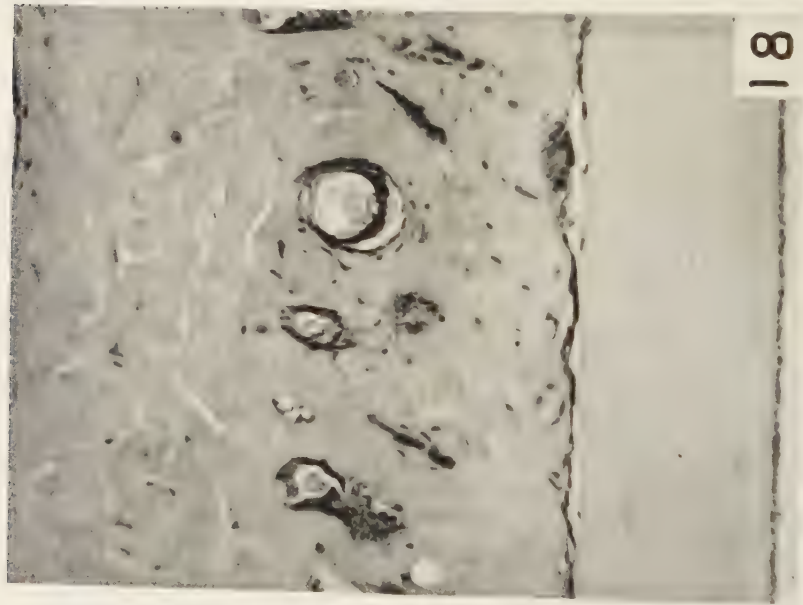


FIGURE 18. Homograft over millipore after 18 days in animal immunized by ordinary graft from same donor 28 days previously. $\times 150$.
FIGURE 19. Part of the same graft as in FIGURE 18 after being replaced on the donor as an ordinary graft for 7 days. $\times 150$.

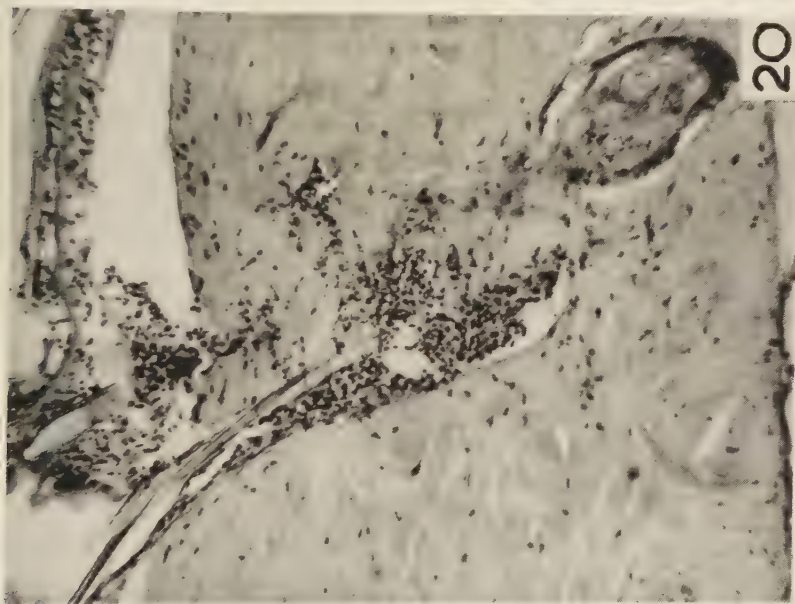


FIGURE 20. Part of the same graft as in FIGURE 18 after being transferred to a new area in the host without membrane for 2 days. $\times 150$. Note the polymorphs in the hair follicle.

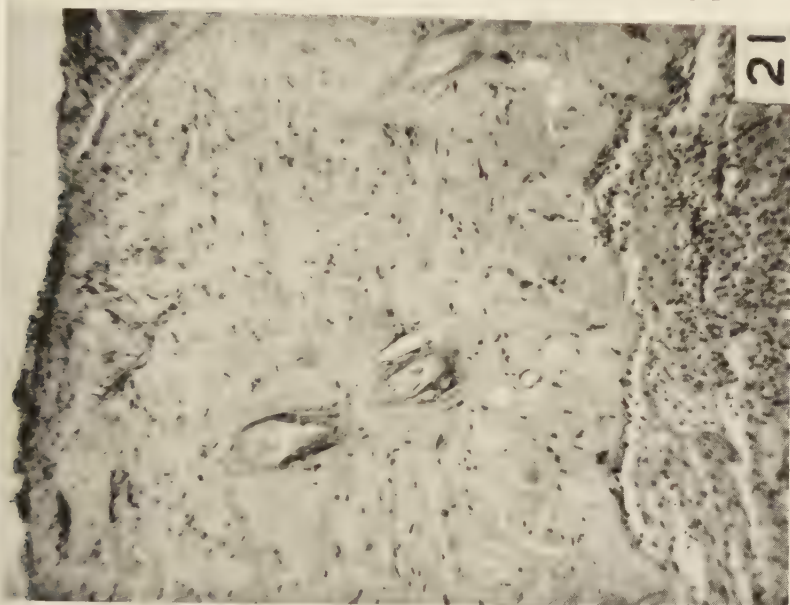


FIGURE 21. Part of the same graft as in FIGURE 18 after being transferred to a new area in the host without membrane for 4 days. Much of the graft appears to have broken down completely, but in other areas not reached by host cells there is some viable-looking epithelium. $\times 130$.

reached by host cells, viable-looking epithelial cells could be seen 3, 4, and sometimes 5 days after transfer. The findings in one pair of animals are illustrated in FIGURES 18 to 21.

Conclusions and Discussion

From the results described it is apparent that split-skin homografts in rats, protected from contact with host cells by millipore membrane, are not effectively antigenic, and, second, that the survival of such homografts is not curtailed by the existence of a state of immunity in the host.

It would seem, however, from the third experiment, that a homograft over membrane in an immunized host is affected in such a way that it is abnormally vulnerable to cellular attack when the protection of the membrane is removed. To test this conclusion further, it is proposed to repeat the experiment in a modified form, leaving the graft over membrane for only 3 or 4 days instead of 13 days. As we have seen, grafts over membrane remain viable for more than 13 days, but their nutrition is certainly impaired. By reducing the period over membrane in the third experiment to 3 or 4 days, regressive changes due to nutritional deficiency would be largely eliminated, but there should still be ample time for humoral antibodies, if they exist, to become fixed to the cells of the graft.

If the conclusion is confirmed, it would appear to follow that humoral antibodies do play a part in the destruction of skin homografts, but that their role is equivalent to that of the opsonins of classical immunology. At present this is put forward only as a hypothesis; more evidence is required before it can be confirmed or rejected. It seems likely to prove fruitful, however, in stimulating further experimental work.

References

- ALGIRE, G. H., J. M. WEAVER & R. T. PREHN. 1954. Growth of cells *in vivo* in diffusion chambers. I. Survival of homografts in immunized mice. *J. Natl. Cancer Inst.* **15**: 493.
- PREHN, R. T., G. H. ALGIRE & J. M. WEAVER. 1955. The diffusion chamber in homograft research. *Transplantation Bull.* **2**: 147.
- PREHN, R. T., J. M. WEAVER & G. H. ALGIRE. 1954. The diffusion chamber technique applied to a study of the nature of homograft resistance. *J. Natl. Cancer Inst.* **15**: 509.
- WEAVER, J. M., G. H. ALGIRE & R. T. PREHN. 1955. The growth of cells *in vivo* in diffusion chambers. II. The role of cells in the destruction of homografts in mice. *J. Natl. Cancer Inst.* **15**: 1737.
- WOODRUFF, M. F. A. & L. O. SIMPSON. 1955. Experimental skin grafting in rats. *Plastic & Reconstr. Surg.* **15**: 451.

Discussion of the Paper

GLENN H. ALGIRE (*National Cancer Institute, National Institutes of Health, Bethesda, Md.*): Woodruff has developed a very useful modification of the diffusion-chamber method for studies of immunity to split-skin grafts. He has presented evidence for skin homografts, as have we (Prehn, Weaver, and Algire) for transplanted sarcomas, that homografts in diffusion chambers do not immunize the host, and that they are not effectively antigenic.

Woodruff has presented evidence, however, that homografts protected by filters from cellular contact in previously immunized hosts are abnormally

vulnerable to cellular attack when the membrane is removed. I am not convinced of this by the evidence so far obtained. One wonders about the condition of these grafts (approximately $400\ \mu$ in thickness) after 13 days within diffusion chambers. In our experience with other cell types, cells appear to be in good condition to a depth of about 8 cell layers against the surface of each filter. Were there controls for these experiments in which skin grafts within diffusion chambers were placed upon isologous rats?

Woodruff has proposed as a hypothesis that humoral substances of the nature of opsonins may have a role, in addition to the role of cells, in bringing about the destruction of homografts. In this connection we have seen no evidence that phagocytosis is a primary event in homograft destruction. Phagocytes come in to "mop up" *after* the cells are dead.

MORRIS K. BARRETT (*National Cancer Institute, National Institutes of Health, Bethesda, Md.*): As I have indicated in the discussion following the paper by R. E. Billingham, this field presents a multitude of contradictions. Therefore it is especially important to seize every opportunity to take note of a thread of connection that might lead to a unifying concept in the future. Such an opportunity has just been presented.

Algire and Woodruff have presented evidence showing that when the antigen (that is, the tissue of the graft) and the host are separated by a millipore filter, no immunity ensues. One presumes that this is because no cells from the graft invade the host. I should have anticipated this because, as I stated in 1951¹ and 1953,² I found that the antigen on the red cell that induces tissue immunity in mice seems to be inseparable from the whole cell or a large piece of it. When the cells or their membranes were broken up into small pieces, the antigen was lost.

Possibly there is a useful unifying principle involved in this similarity of results.

References

1. BARRETT, M. K., W. H. HANSEN & B. F. SPILMAN. 1951. The nature of the antigen in induced resistance to tumors. *Cancer Research*. **11**: 930-935.
2. BARRETT, M. K. & W. H. HANSEN. 1953. Resistance to tumor implantation induced by red cell stromata. *Cancer Research*. **13**: 269-275.

STUDIES ON THE RESPONSE OF THE REGIONAL LYMPH NODE TO SKIN HOMOGRAFTS

By Raymond J. Scothorne

Department of Anatomy, University of Glasgow, Glasgow, Scotland

This paper concerns the changes that occur in the regional lymph node draining a skin homograft and the effect of cortisone on those changes.

The information comes from 3 complementary experiments. The basic procedure was the same in each and is illustrated in FIGURE 1. A full-thickness graft of skin was cut from the ear and exchanged with a similar graft from an unrelated rabbit. Gross and microscopic changes in the lymph nodes draining the operated and control ears and in the spleen were studied. Experiment 1 was concerned with the changes that occur in lymph nodes and spleen in animals receiving no other treatment, while experiments 2 and 3 were concerned with the effect of cortisone on these changes.

The results of experiment 1 have already been published (Scothorne and McGregor, 1955), and can be summarized as follows:

(1) A skin homograft causes a considerable increase in the weight of the regional lymph node draining the graft. This weight increase is not due mainly to the operative procedure, since it is greater in homografted than in autografted animals, nor is it due to the absorption of "toxic products" at the time of graft destruction, since it is already present at 4 days after grafting when the graft is still entirely healthy.

(2) Skin homografts produce no significant weight increase in the spleen.

(3) Neither homografts nor autografts produce any significant increase in weight of the regional node draining the unoperated (control) ear.

In a subsidiary experiment it was shown that lymph drains from the ear to the node of the same side only, and not to the contralateral node.

These findings suggested: (1) that some factor peculiar to the homograft leaves the graft and is carried in the lymphatics to the ipsilateral regional lymph node, and there provokes a weight increase; and (2) that, since the effect is largely confined to the ipsilateral node, little of this so-called "homograft factor" can have reached the blood stream to be generally disseminated.

Study of the histological changes in lymph nodes and spleen fully supported these suggestions. Specific histological changes are, again, mainly confined to the first ipsilateral regional node of animals receiving homografts. In sections, the enlargement of the node is seen principally to involve the cortex and, more particularly, the so-called "tertiary cortical nodules"—that is, the dense cortical lymphatic tissue between and around the secondary nodules or "germinal centers."

Within these enlarged tertiary cortical nodules there are great numbers of cells that are conveniently and rather noncommittally called "large lymphoid cells." They are distributed fairly uniformly throughout the cortex (and, in smaller numbers, in the medullary cords), and do not seem to have any special relationship to the germinal centers.

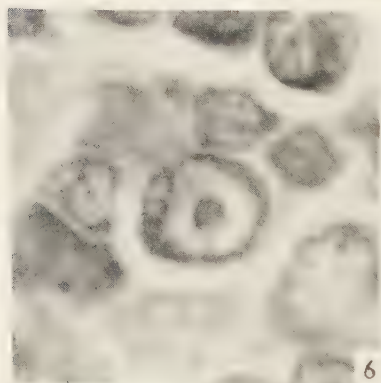
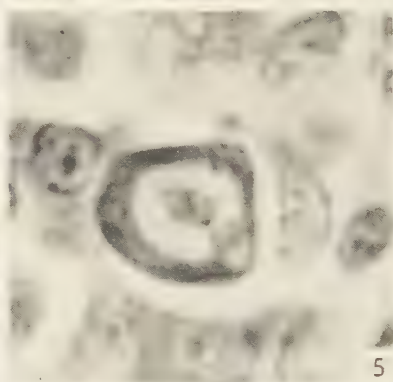
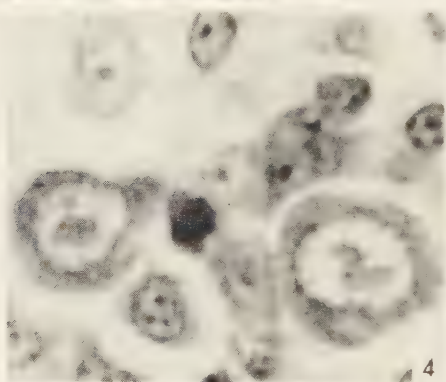
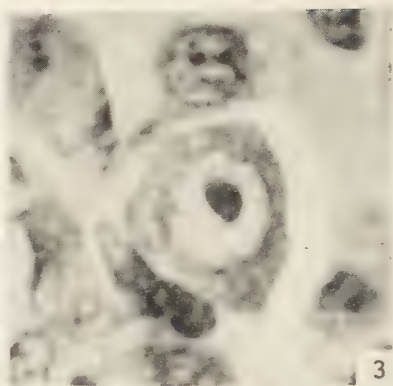
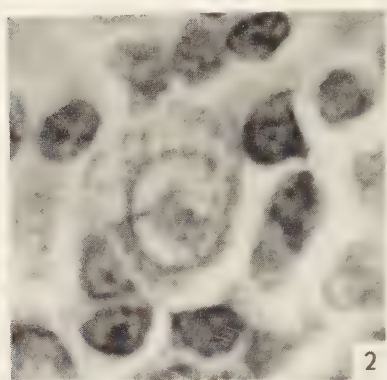
GRAFT 30X15mm.



FIGURE 1. The basic experimental procedure, experiment 1.

Their appearance is somewhat variable. The commoner varieties of form are illustrated in FIGURES 2 to 6. All these cells were photographed in a single section of the regional node of an animal killed on the fourth day after homo-grafting.

The essential characters of the cell are its large size (up to $20\ \mu$), large, pale nucleus, and prominent, basophilic nucleolus, and its basophilic cytoplasm, staining pink or red with pylonin. The cell in FIGURE 2 shows unusually large vacuoles in the cytoplasm, which stained a rather pale pink. In FIGURE 3, the cytoplasm is more basophilic, and the vacuoles smaller but more numerous. The nucleolus is very intensely basophilic. FIGURE 4 shows two large lymphoid cells that may be taken as representative. Lying above the left hand cell is a reticulum cell, the cytoplasm of which is almost invisible in the photograph,



FIGURES 2 to 6. Varieties of the large lymphoid cell in cortex of regional node of rabbit 4 days after homografting. Oil immersion.

FIGURE 7. Large lymphoid cell from medullary sinus of same node. Oil immersion.

but which under the microscope appeared ill-defined, very pale pink, and lace-like in texture. It is not difficult to visualize the transformation, by condensation and increased basophilia of the cytoplasm and nucleolar hypertrophy, of a reticulum cell such as this into a "large lymphoid cell." Proof of such a trans-

formation is still wanting, but studies now in progress in this laboratory seem to indicate that many of the large lymphoid cells do arise from reticulum cells.

FIGURE 5 shows yet another variant of the large lymphoid cell, with compact, strongly basophilic cytoplasm containing a single large vacuole. Finally, FIGURE 6 illustrates one of the smaller members of the cell species, again with a single large vacuole in a strongly basophilic cytoplasm.

So much for the morphology of the large lymphoid cell. It may be emphasized again that this cell is peculiar to the regional node draining a homograft. It is very rare in normal nodes and is unusual in nodes from autografted animals. In homografted animals, the large lymphoid cells are very much more numerous in the node of the operated side than on the control (unoperated) side, and they are absent from the spleen.

The large-lymphoid-cell response is already evident on the third day after grafting, is fully developed by the fourth day, and remains unchanged in character and intensity up to and including the time of graft breakdown between the fifth and eighth days. At 10 days, when the graft is in an advanced state of destruction, the node remains large but the number of large lymphoid cells is very much reduced.

What is the nature of this large lymphoid cell? Those investigators who have interested themselves in the cellular source of antibodies against bacterial and other antigens will have noted resemblances between the large lymphoid cell of the homograft response and the cells that are usually regarded as antibody producers and are described under a bewildering variety of names: (1) the "transitional cell" and "immature plasma cell" of Fagraeus (1948); (2) the "lymphoblast" that Rich, Lewis, and Wintrobe (1939) described as the typical cell of acute splenic tumor; (3) the "blast cell" regarded by Coons and his collaborators as the progenitor of the plasma cell (Leduc, Coons, and Connolly, 1955); (4) the "reticular plasma cells" of Kolouch, Good, and Campbell (1947); and (5) the "young lymphocytes" (of reticulum cell origin) described by Harris and his collaborators (Harris and Harris, 1948).

There is very good evidence that these cells which, if not identical with one another, at least belong to the same general family, do produce antibodies against bacterial and other antigens.

The large lymphoid cell of the homograft reaction appears to belong to this same family of cells, and its appearance in the regional node draining a skin homograft provides anatomical support for the now generally held concept that homograft destruction is the result of an acquired immune response.

The recognition of this cellular reaction represents a small step in the unraveling of the mechanism of homograft destruction. Little can be said at present about how the large lymphoid cell exerts its effect. There are a number of possibilities:

- (1) The cells release antibody into the efferent lymph stream. Earlier attempts to demonstrate serum antibodies against homografts were curiously disappointing, but the work of Billingham and Sparrow (1954) and of Gorer (1955) dispels any doubt of their existence.

- (2) The cells leave the node through the efferent lymphatics, and then reach the graft by way of the blood stream. The cells are certainly very much less

numerous in the node when graft breakdown is advanced, and the cortex gives the impression of having been depopulated of cells. No large lymphoid cells *as such* are found in the graft at any time. Large lymphoid cells—usually appreciably smaller than those in the cortex—are seen in the medullary sinuses (FIGURE 7), but not in such numbers as would account for their paucity in the cortex after breakdown of the graft.

(3) A third possibility is that the large lymphoid cell—the presumed antibody producer—may become transformed into a small lymphocyte and then serve simply as an antibody carrier.

One positive statement can be made about the fate of the large lymphoid cell: that it rarely becomes transformed into a mature plasma cell. It is for this reason that the term “immature plasma cell” seems inappropriate. The plasma cell has attracted a great deal of attention in recent years as the most likely source of antibodies. The present study began as a deliberate search for plasma cells in the regional node. It was only after this search had proved disappointing that my attention was caught by the “large lymphoid cell.” My present feeling is that a great deal of the disagreement about the precise cellular origin of antibodies stems from the application of too rigid a system of terminology. The mature plasma cell probably represents one possible end stage in the life cycle of the antibody-producing cell. It may not even be an obligatory end stage—that is to say, it may develop only in response to a particular intensity and duration of antigenic stimulus. As evidence for this view it may be noted that the plasma-cell concept is championed mainly by those who have studied hyperimmune states.

The next step in the present study was to determine what effect cortisone might have on the development of the large-lymphoid-cell response. Billingham, Krohn, and Medawar (1951a and b) have already shown that cortisone will considerably prolong the survival of skin homografts in rabbits, whether administered systemically in large dosage or applied to the surface of the graft in small dosage.

FIGURE 8 shows the procedure followed in experiment 2, in which the effect of systemic cortisone administration on the development of the large-lymphoid-cell response was studied. The results have been quite clear-cut. The survival of the grafts was prolonged, and the development of the response in the regional lymph node was either suppressed completely or very much reduced (for full details, see Scothorne, 1956).

In experiment 3 an attempt was made to determine how cortisone suppresses the large-lymphoid-cell response and, as a direct corollary, how it prolongs homograft survival. FIGURE 9 illustrates schematically some of the possibilities that must be considered. Starting at the top left of the scheme and working round it counterclockwise, the possibilities are:

(1) *Reduced effective antigenicity of the graft.* Cortisone may act on the graft in such a way as to reduce its capacity to elicit the reaction within the node.

(2) *Reduction in amount of antigen reaching the regional node.* There are good reasons for believing that antigens reach the regional node from the graft principally by way of lymphatics (Scothorne and McGregor, 1955). Cortisone

10mg. CORTISONE DAILY

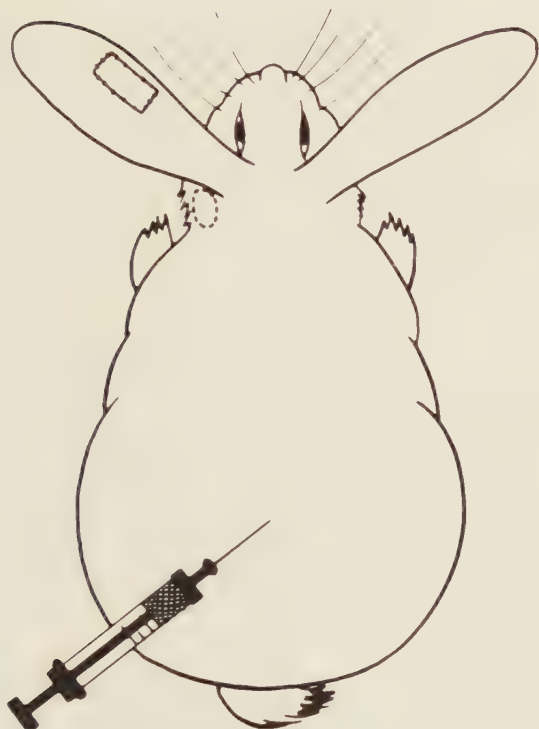


FIGURE 8. Experimental procedure, experiment 2.

might be effective by reducing the rate of lymphatic regeneration within the graft and/or by reducing the permeability of lymphatic endothelium to antigen.

(3) *Inhibition of antibody synthesis within the cells of the regional lymph node.* Quantitative immunochemical methods have shown that cortisone inhibits the antibody response of rabbits and other laboratory animals to known antigens; and it is believed to act by interfering with antibody synthesis rather than by accelerating catabolism of the antibody molecule (see Kass and Finland, 1953, for review). It has also been shown that the usual increase in ribonucleic acid content of the regional node during immunization (Harris and Harris, 1949; Ehrich, Drabkin, and Forman, 1949) is less marked in animals treated with cortisone (Kass and Kendrick, 1952).

(4) *Lysis of antibody-producing cells.* It is well known that cortisone causes atrophy of the lymphatic organs, and it might reduce the antibody response simply by reducing the amount of antibody-producing tissue. Moreover,

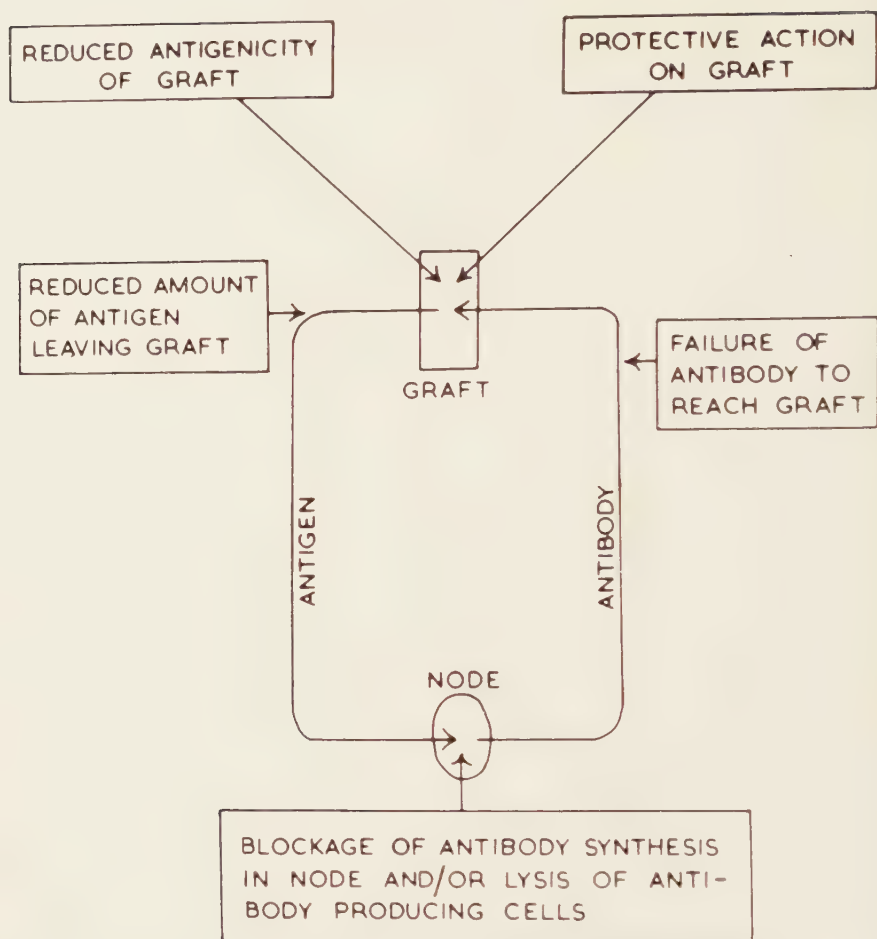


FIGURE 9. Possible mechanisms of prolongation of skin homograft survival by cortisone.

Teilum, Engback, and Simonsen (1950) have shown that cortisone causes disintegration of plasma cells in the spleen of hyperimmunized animals.

(5) *Failure of antibody to reach graft.* Antibody reaches the graft by way of the blood stream, and cortisone might temporarily insulate the graft from circulating antibodies by retarding capillary regeneration within the graft.

(6) *Protective action on the graft.* Cortisone might exert a purely local protective effect on the graft by virtue of its well-known antiphlogistic activity.

These are some of the possible ways in which cortisone might prolong skin homograft survival; still others are mentioned by Toolan (1953 and 1955).

In experiment 3 of the present study, an attempt was made to decide among these various possibilities, and the animals were divided into 2 groups. In group A (FIGURE 10A) cortisone (in the form of a suspension containing 20 mg. ml.) was applied directly to the surface of the graft, while in group B

SURFACE APPLICATION

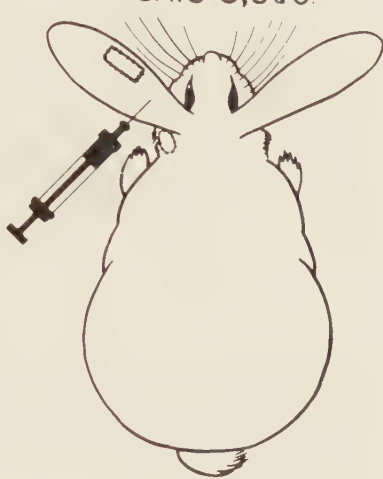
2mg. CORTISONE
DAYS 0, 3 & 6.



a

SUBCUTANEOUS INJECTION

2mg. CORTISONE
DAYS 0, 3 & 6.



b

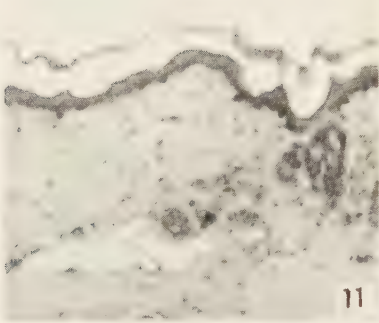
FIGURES 10a and 10b. Experimental procedure, experiment 3.

(FIGURE 10B) it was injected subcutaneously between graft and node. The same small dosage (2 mg. every third day) was used in each case. In group A, it may be assumed that cortisone exerts its maximal effect on the graft; in group B, on the lymph node. The results, presented in detail elsewhere (Scothorne, 1956), seem unequivocal. In group B (the injected group), graft breakdown occurred at the normally expected time of 6 to 8 days, and the regional lymph node showed a fully developed large-lymphoid-cell response. In group A (the local application group), the grafts remained entirely healthy at the time of sacrifice after 6 or 8 days, and the regional node showed no large-lymphoid-cell response.

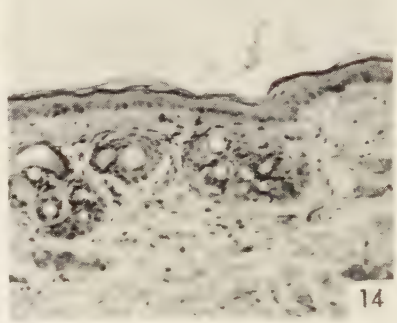
The experiment thus provides further evidence of the prime importance of the large-lymphoid-cell response in homograft destruction. When the response develops, the graft dies; when it does not, the graft survives. But there emerges the interesting supplementary finding that cortisone appears to act primarily upon the *graft*, reducing its capacity for eliciting the immune response, rather than upon the node itself. This finding was entirely contrary to one's expectations, more particularly because of the evidence that cortisone inhibits the synthesis of antibodies against a variety of bacterial antigens.

Cortisone may reduce the effective antigenicity of the graft in two ways:

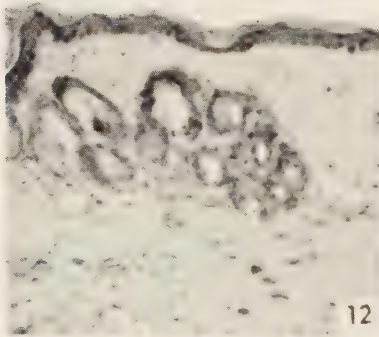
(1) By preventing the very characteristic epidermal proliferation seen in untreated grafts, thereby reducing the total quantity of antigenic material available. This effect is illustrated in FIGURES 11 to 16. FIGURE 11 is a control section of normal rabbit-ear skin. In grafts on animals receiving 10 mg. cortisone daily, the epidermis is little thicker than normal at 4 days after grafting (FIGURE 12) or even at 16 days (FIGURE 13). Epidermal thickening is also



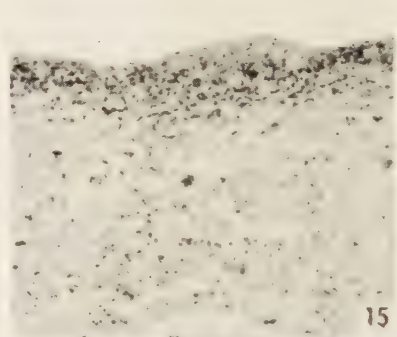
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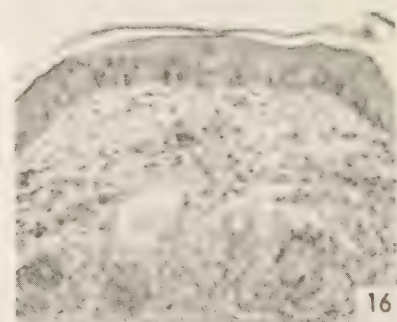
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16

FIGURE 11. Skin of ear, normal control. $\times 130$.

FIGURE 12. Graft of ear skin on host receiving 10 mg. cortisone daily for 4 days. $\times 130$.

FIGURE 13. Graft of ear skin on host receiving 10 mg. cortisone daily for 16 days. $\times 130$.

FIGURE 14. Graft of ear skin on host receiving 2 mg. cortisone locally on days 0, 3, and 6 after grafting. Graft removed at 6th day. $\times 130$.

FIGURE 15. Graft of ear skin, day 6. Host injected between graft and regional node with 2 mg. cortisone on days 0, 3, and 6. $\times 130$.

FIGURE 16. Graft on untreated host, sixth day. $\times 130$.

prevented by local application of cortisone in small dosage (FIGURE 14), but not by injection of the same small amount between graft and node (FIGURE 15). The great thickening of the epidermis of grafts borne by untreated hosts is illustrated in FIGURE 16.

(2) By retarding the regeneration of lymphatics within the graft. Evidence is already at hand (Scothorne, unpublished) of a marked delay in the revascularization of cortisone-treated grafts. It seems probable that there will be a similar delay in the regeneration of lymphatics within the graft. Since all the evidence indicates that antigens leave the graft mainly by the lymphatic route, any interference with lymphatic regeneration must necessarily reduce the antigenic potency of the graft.

Billingham, Krohn, and Medawar (1951a and b) reached a somewhat different conclusion about the precise mechanism of action of cortisone (for full discussion, see Scotthorne, 1956) but we are agreed that cortisone retards the development of the immune response. The reduced large-lymphoid-cell response is the morphological basis of the reduced immune response.

Conclusion

(1) Skin homografts elicit in the cortex of the regional lymph node a specific cellular response, that is, the formation of great numbers of large lymphoid cells.

(2) The large lymphoid cell belongs to the same family of cells that produces antibodies against bacterial and other antigens.

(3) When cortisone is administered systemically in large dosage to the host or is applied in small dosage to the surface of the graft, the large-lymphoid-cell response is suppressed and graft survival is prolonged.

(4) Cortisone appears to act by reducing the effective antigenicity of the graft rather than by reducing the capacity of the regional lymph node to respond to the antigenic stimulus.

References

- BILLINGHAM, R. E., P. L. KROHN & P. B. MEDAWAR. 1951a. Effect of cortisone on survival of skin homografts in rabbits. *Brit. Med. J.* **1**: 1157-1164.
- BILLINGHAM, R. E., P. L. KROHN & P. B. MEDAWAR. 1951b. Effect of locally applied cortisone acetate on survival of skin homografts in rabbits. *Brit. Med. J.* **2**: 1049-1053.
- BILLINGHAM, R. E. & E. M. SPARROW. 1954. Studies on the nature of immunity to homologous grafted skin, with special reference to the use of pure epidermal grafts. *J. Exptl. Biol.* **31**: 16-39.
- EHRICH, W. E., D. L. DRABKIN & C. FORMAN. 1949. Nucleic acids and the production of antibody by plasma cells. *J. Exptl. Med.* **90**: 157-168.
- FAGRAEUS, A. 1948. Antibody production in relation to the development of plasma cells. *Acta Med. Scand. Suppl.* **204**: 5-122.
- GORER, P. A. 1955. The antibody response to skin homografts in mice. *Ann. N. Y. Acad. Sci.* **59**(3): 365-373.
- HARRIS, T. N. & S. HARRIS. 1949. Histochemical changes in lymphocytes during the production of antibodies in lymph nodes of rabbits. *J. Exptl. Med.* **90**: 169-180.
- KASS, E. H. & M. FINLAND. 1953. Adrenocortical hormones in infection and immunity. *Ann. Rev. Microbiol.* **7**: 361-388.
- KASS, E. H. & M. I. KENDRICK. 1952. Effect of cortisone on nucleoproteins of lymph nodes. *Federation Proc.* **11**: 472-473.
- KOLOUCH, F., R. A. GOOD & B. CAMPBELL. 1947. The reticuloendothelial origin of the bone marrow plasma cells in hypersensitive states. *J. Lab. Clin. Med.* **32**: 749-755.

- LEDUC, E. H., A. H. COONS & J. M. CONNOLLY. 1955. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exptl. Med.* **102**: 61-72.
- RICH, A. R., M. R. LEWIS & M. M. WINTROBE. 1939. The activity of the lymphocyte in the body's reaction to foreign proteins, as established by the identification of the acute splenic tumor cell. *Johns Hopkins Hosp. Bull.* **65**: 311-327.
- SCOTHORNE, R. J. 1956. The effect of cortisone on the changes produced in the regional lymph node by a skin homograft. *J. Anat.* **90**: 417-427.
- SCOTHORNE, R. J. & I. A. MCGREGOR. 1955. Cellular changes in lymph nodes and spleen following skin homografting in the rabbit. *J. Anat.* **89**: 283-292.
- TEILUM, G., H. C. ENGBACK & M. SIMONSEN. 1950. Effects of cortisone on plasma cells and reticulo-endothelial system in hyperimmunized rabbits. *Acta Endocrinol.* **5**: 181-193.
- TOOLAN, H. W. 1953. Conditioning of the host. *J. Natl. Cancer Inst.* **14**: 745-765.
- TOOLAN, H. W. 1955. The possible role of cortisone in overcoming resistance to the growth of human tissues in heterologous hosts. *Ann. N. Y. Acad. Sci.* **59**(3): 394-400.

Discussion of the Paper

GUSTAVE J. DAMMIN (*Harvard Medical School, Boston, Mass.*): R. J. Scothorne has reported significant observations on the reaction of lymph nodes to homografts and the effect of locally applied cortisone on the regional lymph node and the skin homograft. The "large lymphoid cell" that is described as part of the specific cellular response to a skin homograft has had many terms applied to it: for example, basophilic lymphoblast. This cell appears in large numbers early in the immune response in the center of the lymphoid follicles in the lymph nodes and the spleen. As this central zone of the follicle increases in prominence, the immediately adjacent zone of small lymphocytes becomes narrower and an outer zone appears. In the course of the immune response, this outer zone becomes more prominent. In the outer zone the predominant cell is larger than the small or mature lymphocyte. It has more cytoplasm and a less dense nuclear chromatin pattern. These predominant cells have been termed lymphoblasts and have been regarded as preplasma cells. Among these cells in the outer zone, however, there are scattered "large lymphoid cells." Therefore, when the immune response is well established, there are 3 distinct zones in the lymphoid follicle: (1) an inner zone I dominated by reticulum cells and "large lymphoid cells"; (2) an intermediate zone II in which the mature small lymphocyte is preponderant; and (3) an outer zone III which contains numerous lymphoblasts and some "large lymphoid cells." The origin of the prevailing cell of zone III is in doubt, since it is at the periphery of this zone and, of course, in the medullary portion of the lymph node and the red pulp of the spleen that plasma cells are found when antibody synthesis is active.

The following questions must be answered: (1) How specific is the zoning phenomenon of the lymphoid follicle? (2) What is the role of the "large lymphoid cell" in the immune response?

We have observed the zoning phenomenon in the course of an immune response and in conditions in which it may reflect an immune response, for example, thrombocytopenia purpura and terminal stress reactions with infections. Under these circumstances, plasma cells are usually prominent in the spleen, lymph nodes, and marrow. The zoning phenomenon may represent a morphologic manifestation of an immune response, and the "large lymphoid cell" may have a role in this phenomenon. The synthesis of antibody in zone I,

however, in which the "large lymphoid cell" is a prominent cell, has not been demonstrated to be as active as in other portions of the lymph node and spleen. With a fluorescent label on the antigen, antibody synthesis has been found more active in medullary areas of lymph nodes and in the trabecular portions of the red pulp of the spleen.

It has been postulated that the "large lymphoid cell" is concerned with a modification or preparation of the antigen necessary before other cells, that is, plasma cells and possibly lymphoblasts, may respond with antibody synthesis. Such a role is suggested by Scothorne's work, namely, that by suppressing the "large-lymphoid-cell" response, a step necessary for prompt antibody synthesis is removed and the survival of the skin homograft is thereby prolonged. It seems more likely, however, that several types of cells, possibly arising from the same parent cell but, in a number of sites, in the reticuloendothelial tissues, are capable of antibody synthesis, and that all may be affected in some degree by adrenal cortical steroids, ionizing radiation, and other agents that affect the immune response.

STUDIES ON THE HOMOTRANSFER OF SUSPENSIONS OF LYMPH-NODE CELLS*

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In recent years some of the studies on the formation of humoral antibody have employed the technique of transfer of cells, and have thus entered the area of tissue transplantation and encountered some of the problems that are met in the homotransplantation of organized tissues. This approach can be considered to have developed from earlier studies of Landsteiner and Chase¹ on delayed hypersensitivity. In 1942, these investigators found that hypersensitivity to simple chemical compounds could be conferred on normal guinea pigs by the transfer of cells of peritoneal exudates of sensitized guinea pigs. In 1945 Chase² described the transfer to normal guinea pigs of hypersensitivity to tuberculin by cells of peritoneal exudates, spleens, and lymph nodes of guinea pigs hypersensitive to tuberculin. In 1950, the same investigator reported that by the transfer of cells of spleens and lymph nodes of donor guinea pigs with skin sensitivity and high titers of anaphylactogenic antibodies, both of these properties could be conferred on the recipient animals.^{3, 4} Since then, the technique of homotransfer of cells of the lymphatic system has been used increasingly in the study of formation of humoral antibodies to several antigens.⁵⁻⁸

In our laboratory, studies have been carried out on antibody formation to the agglutinin of *Shigella paradyserteriae*, involving the transfer of cells from lymph nodes of donor rabbits to recipient rabbits. In earlier studies of this series, these organisms were injected into the foot pads of donor rabbits; 4 days later, at a time when the concentration of agglutinins in the draining lymph nodes was approaching its peak, cells teased from the nodes were transferred to recipient rabbits. The recipients were then bled periodically and the sera were tested for agglutinins to dysentery bacilli. It was found that agglutinins appeared on the first day after transfer, rose in titer to a maximum on about the third day, and began to decline after the fifth day. Typical curves are shown in FIGURE 1.

It soon became apparent that viable cells were necessary for this sequence of events, since the transfer of cells injured in any one of a number of ways—by alternately freezing and thawing, by heating to 50° C., or by maintaining them at 37° C. for 24 hours—was not followed by the appearance of agglutinins in the sera of recipients in the first few days after transfer.⁹ Such experiments are shown in FIGURE 2. As can be seen in this figure, the transfer of injured cells may be followed by the appearance of agglutinins later and in a lower

* This investigation was supported by Research Grant H-869 from the National Heart Institute of the National Institutes of Health, United States Public Health Service, Department of Health, Education, and Welfare, Bethesda, Md.

FIGURE 2 and part of FIGURE 4 are reproduced from *The Journal of Immunology*, Baltimore, Md.; FIGURE 3 and part of FIGURE 6 from *The Journal of Experimental Medicine*, Baltimore, Md.; and the remainder of FIGURE 4 from the *Proceedings of the Society for Experimental Biology and Medicine*, by permission of the respective publishers.

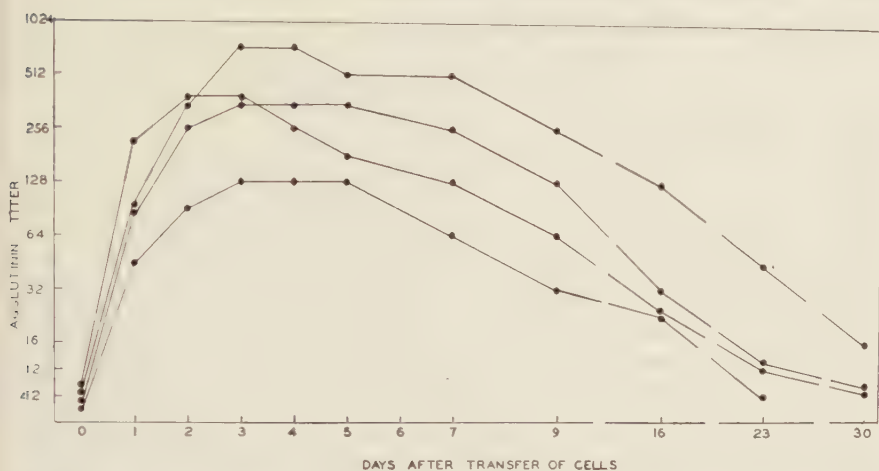


FIGURE 1. Appearance of agglutinins to dysentery bacilli in the sera of recipient rabbits following the transfer of lymph-node cells obtained 4 days after the injection of bacilli into the foot pads of donor rabbits.

EFFECT OF VARIOUS TREATMENTS OF CELLS ON ANTIBODY TITER OF RECIPIENTS

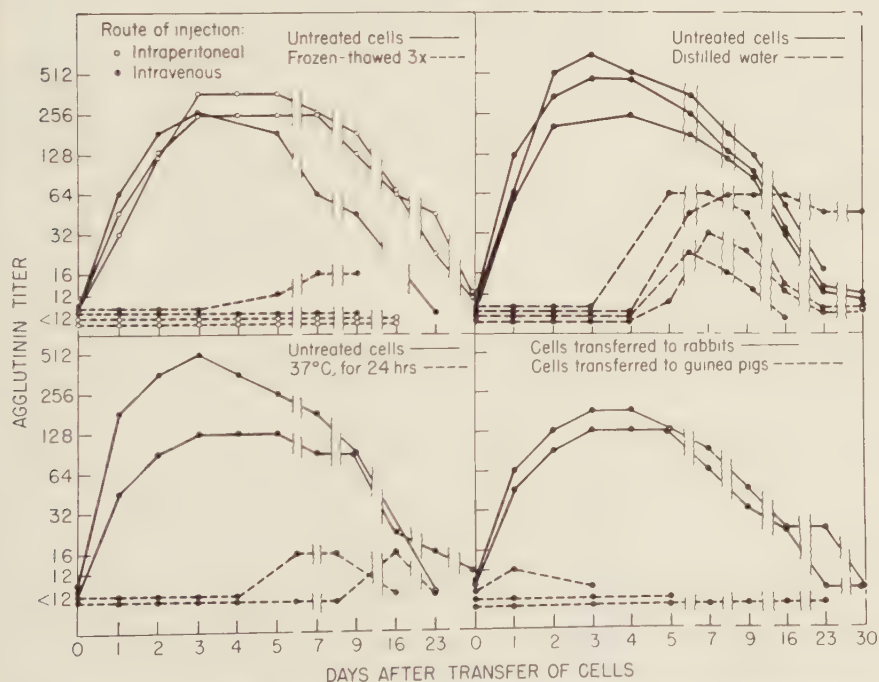


FIGURE 2. Agglutinin titers in the sera of recipient rabbits following the transfer of lymph-node cells injured by various means, in comparison with control suspensions of cells.

range of titers. This pattern of antibody titers was considered to reflect active "immunization" by antigenic material carried over in the cell suspension.

In later studies, one of the effects investigated was that of variations in the interval between the injection of the antigen into the donor animal and the collection of its lymph-node cells for transfer.¹⁰ It was found that, even if this interval were decreased from 4 days to as little as 10 minutes, homologous agglutinins appeared in the sera of irradiated recipients, although somewhat later, as will be shown presently. In the next extensions of these observations, it was found that lymph-node cells obtained from rabbits that had not been previously injected with *Shigella* could be incubated *in vitro* with suspensions of these organisms or with soluble material derived from them and transferred to X-irradiated recipient rabbits, with the subsequent appearance of agglutinins in the sera of these animals.^{11, 12} Studies in the latter system will be referred to as "*in vitro* incubation" experiments, in contrast to the "donor-injection" system referred to in the preceding paragraph. The body of this report describes comparative observations made in the 2 systems.

Time curves of antibody concentration in the sera of recipient rabbits. In the donor-injection system, the relation of the time of appearance of agglutinins in the recipients to the time of transfer of cells was found to be a function of the interval allowed between the injection of antigen into the donor and the collection of its lymph-node cells. The time curve of antibody concentration corresponding to a 4-day interval between injection of antigen and sacrifice of the donor was shown in FIGURE 1, and is illustrated also in one part of FIGURE 3. In the case of a 3-day interval between injection of the donor and collection of its cells, the antibody curve of the recipients was quite similar. Where the donor interval was only 2 days, however, agglutinins did not appear above the present threshold of measurement on the first day after transfer, but rather on the second day; in the case of a 1-day donor interval, agglutinins appeared on the third day after cell transfer. Thus, with decreasing values of the donor interval, agglutinins appeared in sera of recipients of untreated cells progressively later with respect to the time of cell transfer. On the other hand, as can be seen in FIGURE 3, recipients of heated cells showed agglutinins earlier and in higher titer with decreasing values of the donor interval. The time of appearance of agglutinins after the transfer of heated cells was consistent with the interpretation that this was due to active "immunization" of the recipients by antigen carried over in the cell suspension. In the case of 4-, 3-, and 2-day donor intervals it was possible to distinguish between the effect of the transfer of untreated and of injured cells. As the interval decreased to 1 day, however, agglutinins appeared on the third day after transfer of cells, whether these were untreated or injured, as can be seen in the upper left part of FIGURE 3, so that it was impossible to differentiate between the 2 effects. In order to obviate the possibility of active "immunization," some recipient rabbits were subjected to deep Roentgen irradiation which, in adequate dosage, is known to suppress the ability of the animal to form antibody actively. When this procedure was employed, it became possible to interpret data obtained with short donor intervals, and, as noted above, a donor interval

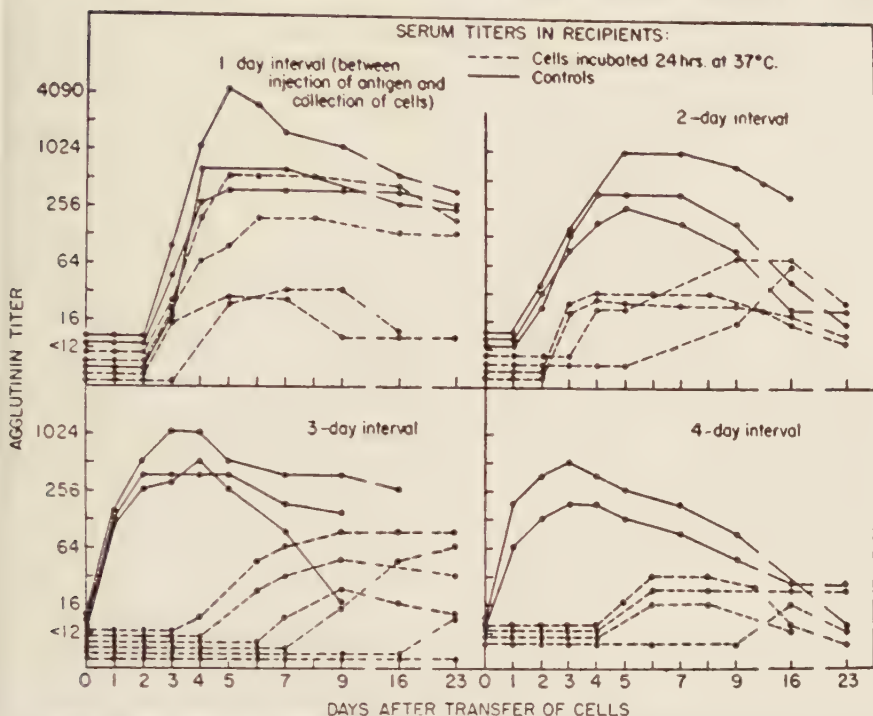


FIGURE 3. The appearance of agglutinins to *Shigella paradysenteriae* in the recipients of untreated and injured cells of lymph nodes obtained 1, 2, 3, and 4 days after the injection of antigen into the foot pads of donors.

of as little as 10 minutes yielded cells the transfer of which was followed by the finding of agglutinins in the sera of irradiated recipient rabbits.

In the case of *in vitro* incubation of lymph-node cells with antigenic material, time curves of agglutinins were observed in the sera of irradiated recipients such as those shown in FIGURE 4. This figure includes data obtained on incubation of cells with suspensions of whole organisms or, alternatively, with rabbit serum in which the dysentery organisms had previously been incubated. It can be seen that agglutinins appeared at the present threshold of measurement on the fourth day after cell transfer, then rose, and later declined in titer in a pattern similar to that observed in the donor-injection experiments. Irradiated recipients of heated cells did not develop agglutinins within the first week after transfer. Thereafter, agglutinins were occasionally observed in these animals in slowly rising titer, probably reflecting some degree of recovery of these recipients from the effects of irradiation on their capacity to form antibodies actively. In experiments involving the use of soluble antigenic material, this late appearance of antibody occurred only rarely, presumably because of more effective removal of excess antigen after the *in vitro* incubation with the lymph node cells.

Effects of injurious treatments of the cells. As noted before, when cells were obtained from donor rabbits injected with dysentery organisms and subjected

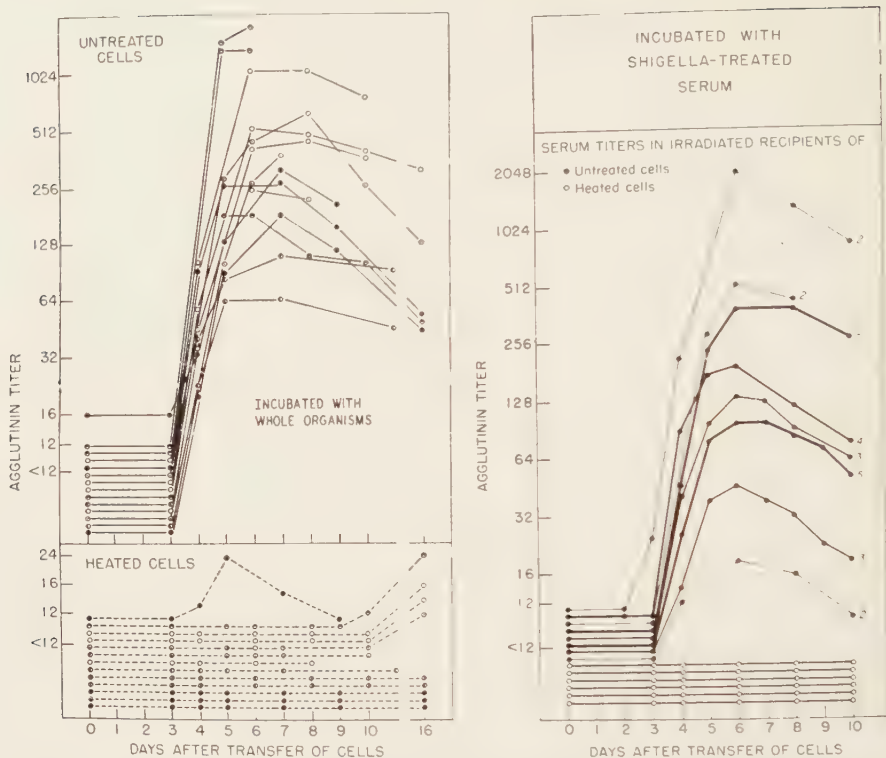


FIGURE 4. Serum-agglutinin titers of irradiated recipients of untreated and heated lymph-node cells. In the experiments illustrated on the left, the cells had been incubated *in vitro* with dysentery bacilli prior to transfer. The various symbols indicate different experiments. In the experiments illustrated on the right, the cells had been incubated with *Shigella*-treated serum prior to transfer. The geometric-mean titers of groups of rabbits with similar titers are shown, with the number of rabbits represented in the curve indicated, and the thickness of the solid line is proportional to the number of recipients.

to various forms of injurious treatment before transfer, the recipients failed to show agglutinins to these organisms in the usual pattern. In experiments involving *in vitro* incubation, cells were also subjected to injury by heating, by sonic oscillation, or by suspension in distilled water; the recipients of such cells again failed to develop serum-agglutinin titers. Some of the data obtained in such experiments are shown in FIGURE 5.

Effect of irradiation of recipient rabbits prior to transfer. As was indicated above, irradiation of recipient animals had been employed to suppress active formation of antibodies by the host tissues, and thus to make possible differentiation between the effects of active "immunization" and those of cell transfer. It was found that whereas irradiated recipients of heated cells generally did not develop agglutinins in the first week after transfer, the recipients of untreated cells developed agglutinins at the usual level or higher. In comparing the antibody curves and maximum agglutinin titers of a large group of irradiated and normal recipients in the donor-injection system, it was found that the peak geometric mean titer of irradiated recipients was more

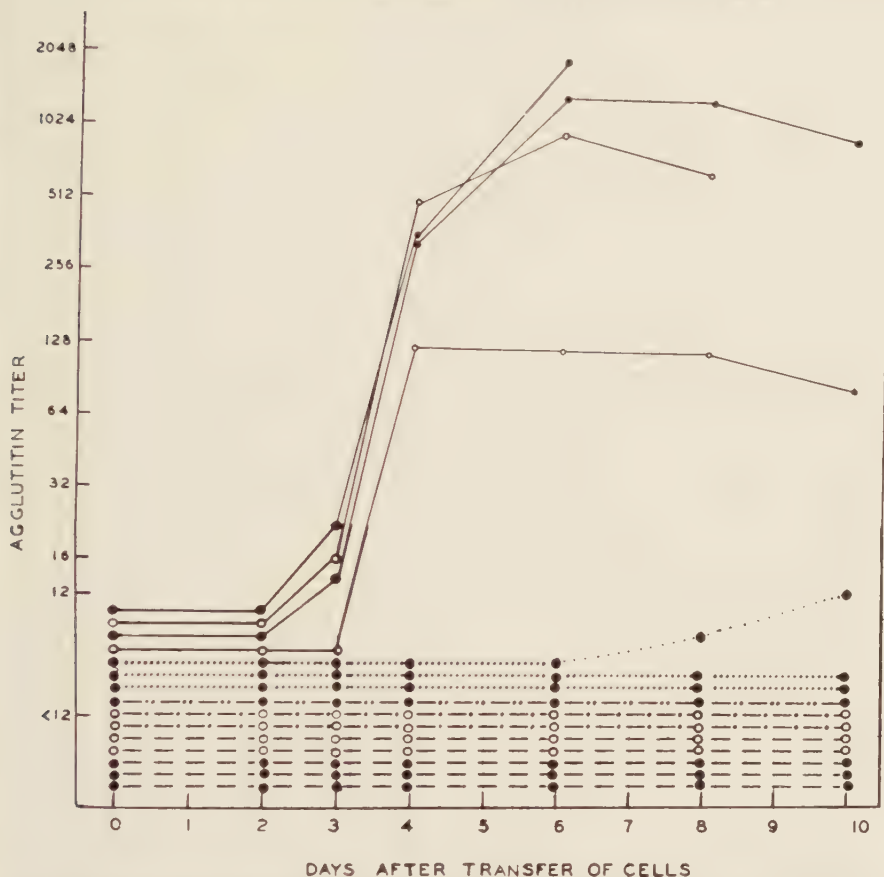


FIGURE 5. Serum-agglutination titers of irradiated recipients of lymph-node cells incubated *in vitro* with soluble antigenic material derived from dysentery bacilli. Represented are recipients of untreated cells and of cells treated in various ways: untreated cells ———; cells suspended in distilled water ----; sonic-oscillated suspension; supernate of sonic-oscillated suspension; sediment of sonic-oscillated suspension

than 4 times as high as that of the normal group. In the *in vitro*-incubation system, the irradiated recipients again showed higher agglutinin titers than did normal recipients, and to quite a similar degree, as can be seen in FIGURE 6, although other differences, such as the time of appearance of antibody, are evident.

Species specificity. In a few of the donor-injection experiments, cells of rabbits had been transferred to guinea pigs with no apparent effect. Such experiments were repeated in the *in vitro* system, and again no antibody was found in sera of guinea pig recipients, although rabbit recipients of such cell suspensions yielded the usual results.

It had been observed that irradiated recipients in homotransfer experiments developed higher agglutinin titers than normal ones, and it had been considered possible that this was due to decreased reaction by irradiated tissues to cells

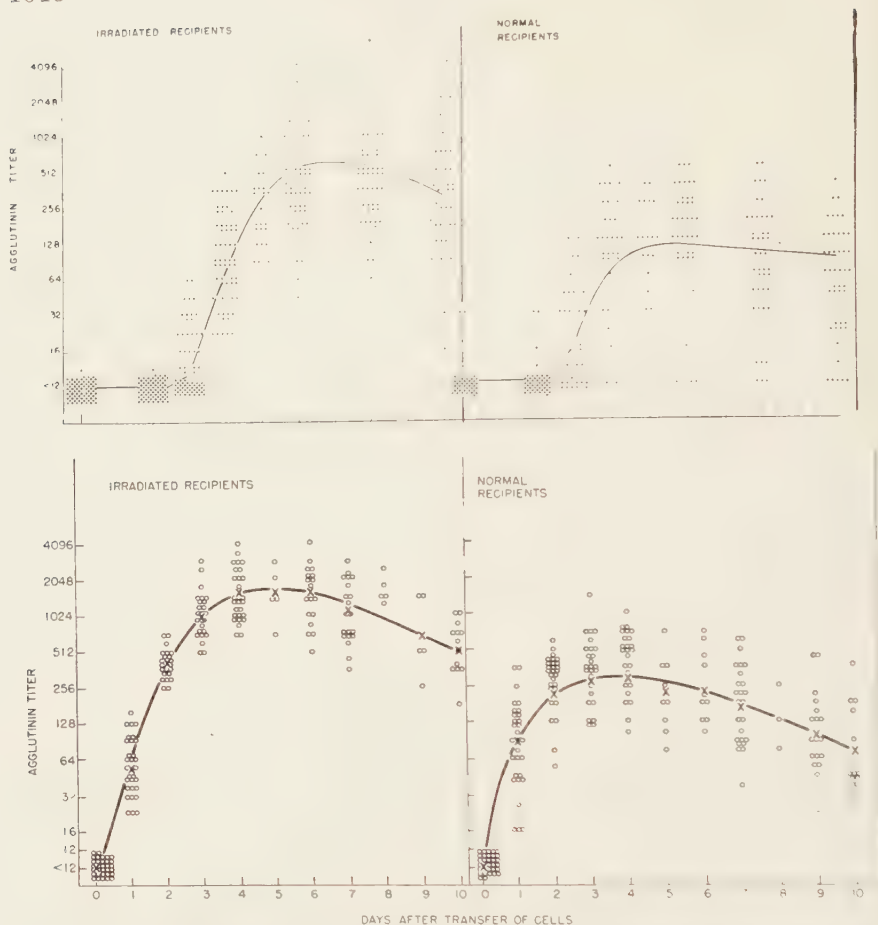


FIGURE 6. Serum-agglutination titers of normal and irradiated recipients. Each circle or dot represents the titer of an individual recipient on the day indicated by the abscissa. The solid lines connect the geometric-mean titers obtained on each day within each of the groups of recipients. Data in the lower part of the figure are from experiments involving cells obtained 3 days after the injection of dysentery bacilli into the donors. Data in the upper part of the figure are from experiments in the *in vitro* incubation system.

that might have tissue incompatibilities within a given species. On the assumption that the reaction of host tissues to heterologous cells might be of a nature similar to, but of greater degree than, that involved in homologous transfer, experiments in which guinea pigs were irradiated at the maximum dosage level compatible with 1-week survival were undertaken. Lymph-node cells were obtained from rabbits injected 3 days earlier with typhoid bacilli and were then incubated *in vitro* with soluble forms of the dysentery agglutino-gen. These cells were transferred to rabbits irradiated at the usual level of 425 r, to normal guinea pigs, and to guinea pigs irradiated at levels of dosage up to 1000 r. The guinea pigs had been obtained from 2 sources and were irradiated at levels that, within each group, caused death in 75 per cent of the guinea pigs within 8 days. In addition, in the last few experiments

TABLE 1
SERUM AGGLUTININ TITERS OF GUINEA PIG AND RABBIT RECIPIENTS OF RABBIT LYMPH-NODE CELLS
Recipients of rabbit lymph node cells

			Guinea pigs												Rabbits (all irradiated, 425 r)			
			Serologic tests												Serologic tests			
X-ray dose, r	Cortisone	Source*	Number of expts.	Number of recipients	Maximum titer in recipients												Number of recipients	Maximum titer in recipients
					Dysentery						Typhoid							
					<12	12	16	24	32	48	64	<12	12	16	24			
0		D B	4 2 1†	15 9 2	12 3 1	2 1 1		1 1 1	2	1 2	1	15 4 2	3 1 2	1 1 1	4	2-256 1-768 1-2048	1-96 1-128 1-192 1-256	
400		D	2	15	13		1	1				15			2	1-384 1-1024 1-384	1-128 1-384	
600		D	2	12	11		1					11	1		6	1-128 2-192 1-256 1-384 1-512	2-64 1-96 3-128	
		B	1	4	3	1						4						
800		D B B	2 1 1	12 4 4	11 3 4			1				12 3 3		1	3	2-256 1-2048 1-512	1-96 1-128 1-512	
1000	X	D D B	4 2 1	24 10 3	22 10 2			2				22 10 3	1	1	2	1-384 1-768 1-192	1-96 1-128 1-192	

* Source: D. Detweiler, R. B. Bolton Farms

* Source: D, Detweiler, R.; B, Bolton Farms.
† Healed cells transferred.

half of the irradiated guinea pigs were also treated with 20 mg. of cortisone every second day from 2 days prior to irradiation to the end of the experiment. In these experiments, the guinea pigs weighed, on the average, half as much as the rabbits, but the number of cells transferred to guinea pigs was always the same or twice as great as that transferred to rabbits. The sera of all recipients were tested for both typhoid and dysentery agglutinins. The data obtained, summarized in TABLE 1, show that the transfer of rabbit lymph-node cells to guinea pigs failed to result in the appearance of appreciable titers of agglutinin in the guinea pig recipients, regardless of the level of irradiation and of cortisone treatment. In the groups of normal guinea pig recipients, agglutinins appeared on occasion, but since agglutinins also appeared in normal recipients of heated cells to the same extent and at similar times, it was felt that this could be attributed to active "immunization" brought about by antigen present in the cell suspension that was transferred.

Cytologic observations. The cell populations of samples taken from 14 of the donor-injection and 15 of the *in vitro*-incubation types of experiments were studied by smears stained with May-Gruenwald-Giemsa's and Wright's stains. On each slide, approximately 400 cells were counted and classified. In both types of experiments, the predominating cells were those of the lymphocytic series. In the case of the cell suspensions used in the donor-injection experiments, the average percentage was 95.2 and, in the *in vitro* experiments, the average percentage of cells of the lymphocytic series was found to be 99. These data are shown in TABLE 2.

Discussion

The observations reported here indicate that cells obtained from lymph nodes of donors previously injected with dysentery bacilli or obtained from lymph nodes of noninjected donors, but incubated *in vitro* with dysentery

TABLE 2
PERCENTAGE DISTRIBUTIONS OF CELL TYPES IN SMEARS OF CELL SUSPENSIONS
IN STUDIES INVOLVING THE TRANSFER OF LYMPH-NODE CELLS

Cells	Type of Experiment			
	Donor-injection		<i>In vitro</i> incubation	
	Per cent		Per cent	
	range	average	range	average
Lymphocytic series				
Mature (small)	52.8-84.2	72.4	[90.0-97.0	[92.6
Medium	7.8-28.4	14.4		
Large	3.2-15.9	7.0		
Prolymphocyte	0.3- 2.3	1.2	3.5-6.1	5.3
Lymphoblast	0.1- 1.0	0.3		
Total		95.3	0-1.5	99.0
Plasma cell		1.9		.6
Other		2.8		.4

bacilli or soluble products derived from them, can be transferred to fresh animals of the same species, with subsequent appearance in the sera of the recipient of agglutinins to dysentery organisms; the time curve of concentration of these agglutinins falls into a pattern characteristic for each of the 2 types of experiments. An understanding of the source of the antibody found in the recipient after the transfer of the lymph-node cells would be important both in relation to the problems of antibody formation and because of the possible implications for the problems of tissue- or cell-transplantation.

In discussing the source of the antibody that appeared in the recipients of donor-injection experiments, we must consider the following possibilities: first, that the antibody may have been formed in the donor and released in the recipient from the cells transferred; second, that antigen present in or among the cells may have given rise to the active formation of antibody by the tissues of the recipient; or, third, that cells that are transferred and in which the process of antibody formation has been initiated may continue their synthetic function in the new host.

The data presented provide evidence against the possibility that either preformed antibody or antigen is responsible for the antibody found in the recipient. The fact that the transfer of cells that are heated or incubated at 37° C. for 24 hours does not give rise to the appearance of agglutinins indicates the necessity for viable cells, and one might expect that release of preformed antibody would not be prevented by injury of the cells. The same consideration also applies to the role of the antigen. Since the injurious forms of treatment mentioned above do not affect the antigenicity of the agglutinin, one would not expect so different a response on injuring the cells before transfer if active "immunization" by transferred antigen accounted for the antibody found. (That some native antigen is, in fact, present in the cell suspensions is indicated by the late appearance of agglutinins in low titer after the transfer of heated cell suspensions. The pattern of the antibody curve seen under the latter circumstances is different from that seen after the transfer of fresh cells.) In addition, when recipients were irradiated, prior to transfer, at a dosage level designed to suppress the animal's capacity actively to form antibodies, the agglutinin titers of these animals after transfer were not reduced, but were, in fact, increased.

In the *in vitro*-incubation system, the question of preformed antibody does not arise, since the donors are not injected with antigen. The question here is only whether the antibody found in the recipient could be due to active "immunization" by the recipient. In this system, the recipients were irradiated, prior to transfer, at a level calculated to suppress the host's ability to form antibody actively. Here again the transfer of heated cells was not followed by the appearance of agglutinins, in contrast to the effect of the transfer of untreated cells. Also, since soluble products of dysentery bacilli were used, the amount of antigen remaining in the suspension after incubation and the subsequent washing of the cells could be reduced to such a point that a high percentage of normal recipients of heated cells failed to show the development of antibodies.

If, therefore, viability of the cells is necessary for the effect described here, it must then be considered possible that the transferred lymph-node cells continue to function in their new host and thereby, in some direct or indirect manner, constitute the source of the antibody found. The relative contribution of the transferred cells and that of the host tissues cannot be evaluated at present, but the fact that higher levels of agglutinin were found in irradiated than in normal recipients would imply a relatively passive role, in immunologic terms, for the host tissue.

If the transferred cells continue to function in their new host, it is conceivable that these cells are responsible for the antibody, or that these cells release some substances essential to the process of antibody formation that can be used by the irradiated host tissues to produce antibody. A hypothesis of the latter type is suggested by Jacobson and his co-workers,¹³ who reported experiments in which rabbits were exposed to 500 or 800 r, with the spleens shielded during the treatment. The spleens were left intact for 24 hours, after which they were removed surgically. If, 24 hours after splenectomy, sheep erythrocytes were injected into these animals, it was found that hemolysins to sheep erythrocytes were subsequently produced. The authors attribute this to a restoration of the functional capacity of the irradiated rabbits' antibody-forming cells by a humoral substance entering the general circulation of the animal from the shielded spleen during the 24-hour period prior to splenectomy. It is not possible, however, on the basis of the data presented in that study, to deny any role to cells entering the circulation of the irradiated animal from its shielded spleen, which thus, in effect, would provide the irradiated animal with a transfusion of cells from its own nonirradiated lymphatic tissue.

In our experiments, the results also do not provide direct data as to whether the cells transferred are the sites of synthesis of the antibody found in the sera of the recipient animals or whether these cells provide some material that makes possible the synthesis of antibody by the irradiated tissues of their new host.

Summary

If lymph nodes draining sites of injection of *Shigella paradysenteriae* are teased and the suspension of cells thus obtained is transferred to fresh recipient rabbits, agglutinins to *Shigella* appear in the sera of the latter. Alternatively, if lymph-node cells obtained from normal rabbits are incubated *in vitro* with suspensions of *Shigella* or with soluble antigenic materials derived therefrom, and are then transferred to irradiated recipient rabbits, agglutinins appear in the sera of the latter.

The following observations have been made in both of these experimental situations: injury to the transferred cells results in failure of appearance of agglutinins; previously irradiated recipients of such cells show higher serum concentrations of agglutinins than normal recipients of similar suspensions; and no production of antibody can be observed when cells from rabbit donors are transferred to guinea pigs, even when the latter are subjected to irradiation and cortisone therapy at maximal levels of dosage compatible with 1-week survival.

Acknowledgment

We are grateful to the staff of the Radiology Department of the Graduate Hospital of the University of Pennsylvania for its generosity in permitting us the use of their X-ray therapy machine throughout this study.

References

1. LANDSTEINER, K. & M. W. CHASE. 1942. Experiments on transfer of cutaneous sensitivity to simple compounds. *Proc. Soc. Exptl. Biol. Med.* **102**: 61.
2. CHASE, M. W. 1945. The cellular transfer of cutaneous hypersensitivity to tuberculin. *Proc. Soc. Exptl. Biol. Med.* **59**: 134.
3. CHASE, M. W. 1951. Development of antibody following transfer of cells taken from lymph nodes of sensitized or immunized animals. *Federation Proc.* **10**: 404.
4. CHASE, M. W. 1953. Immunological reactions mediated through cells. *In* A. M. Pappenheimer, Jr. *The Nature and Significance of the Antibody Response*. Columbia Univ. Press. New York, N. Y.
5. HARRIS, S. & T. N. HARRIS. 1951. Transfer of cells from lymph nodes of rabbits following regional injection of antigens. *Federation Proc.* **10**: 409.
6. WAGER, O. A. & M. W. CHASE. 1952. Appearance of diphtheria antitoxin following transfer of cells taken from immunized rabbits. *Federation Proc.* **11**: 485.
7. STAVITSKY, A. B. 1954. Participation of the popliteal lymph node and spleen in the production of antitoxin in the rabbit. *J. Infectious Diseases.* **94**: 306.
8. ROBERTS, J. C. & F. J. DIXON. 1955. The transfer of lymph node cells in the study of the immune response to foreign proteins. *J. Exptl. Med.* **102**: 379.
9. HARRIS, S., T. N. HARRIS & M. B. FARBER. 1954. Studies on the transfer of lymph node cells. I. Appearance of antibody in recipients of cells from donor rabbits injected with antigen. *J. Immunol.* **72**: 148.
10. HARRIS, S. & T. N. HARRIS. 1954. Studies on the transfer of lymph nodes. III. Effects of variation in the interval between the injection of antigen into the donor and collection of its lymph node cells. *J. Exptl. Med.* **100**: 269.
11. HARRIS, S. & T. N. HARRIS. 1954. Studies on the transfer of lymph node cells. V. Transfer of cells incubated *in vitro* with suspensions of *Shigella paradyenteriae*. *J. Immunol.* **74**: 318.
12. HARRIS, T. N., S. HARRIS & M. B. FARBER. 1955. Studies on the transfer of lymph node cells. VI. Transfer of cells incubated *in vitro* with *Shigella*-treated rabbit serum. *J. Immunol.* **75**: 112.
13. JACOBSON, L. O. & M. J. ROBSON. 1952. Factors effecting X-ray inhibition of antibody formation. *J. Lab. Clin. Med.* **39**: 169.

Discussion of the Paper

H. SHERWOOD LAWRENCE (*New York University College of Medicine, New York, N. Y.*): The Harris' interesting studies are pertinent both in their purely immunological implications and in their relation to homotransplantation phenomena. The observations underline the import of Chase's¹ suggestion that the mode of preparation of the donor of the cells conditions the effects that are transferred and, thereby, the specific type of immunological response of the recipient. In these experiments the donor animal is carefully stimulated to produce and thus transfer only serum antibody. The recipient animal is also carefully prepared as a passive receptacle to allow the continued functioning of the antibody-producing mechanism carried over by the cells while in residence. Such preparation precludes any active contribution of the recipient to the mechanism transferred.

Chase¹ demonstrated that cells from donor animals deliberately sensitized were capable of simultaneously conferring upon recipients delayed dermal sensitivity and serum antibody formation to the same test material. This finding has been recently observed by Mitchison and Dube² following the

cellular transfer of the accelerated homograft rejection phenomenon. Here it was shown that the same cells that mediated the transfer of accelerated rejection of homografts also mediated at the same time the recipient's capacity to produce hemagglutinating antibody.

A curious and ill-understood aspect of this general problem is the recipient's apparent tolerance of homologous lymph-node cells without interference to the mechanism of transfer. As the Harrises have pointed out, transfer from species to species using heterologous cells has not been successful. This has also been the experience of Chase.³ Moreover, Rammekamp⁴ has not been successful in attempts to transfer delayed tuberculin hypersensitivity from man to guinea pig using peripheral blood leukocytes, nor have we been successful in our attempts to do so.⁵ A possible exception to this apparent species barrier has been reported by Kourilsky *et al.*⁶ for the transfer of tuberculin sensitivity from man to guinea pig, using peripheral blood leukocytes.

A provocative finding is the observation that as little as 10 minutes *in vitro* contact between antigen and cells from an unprepared donor is sufficient to induce a mechanism whereby such cells are then capable of producing serum antibody in residence in the recipient. Chase³ has suggested that the events that result in the induction of delayed bacterial hypersensitivity very likely occur much earlier in time than we are accustomed to believe. This concept is supported by Freund and Lipton's⁷ observation that excision of an adjuvant-tissue injection site as early as one hour after injection does not prevent either the development of allergic encephalomyelitis or sensitization to tuberculin. Further evidence of a general biological nature to support this thesis is suggested by the almost instantaneous occurrence of the fertilization process following contact between a mammalian ovum and spermatozoon. The far-reaching complexities of the biological and biochemical events that are initiated in that instant in time are well-documented.

References

1. CHASE, M. W. 1952. The allergic state. *In* Bacterial and Mycotic Infections of Man. R. J. Dubos, Ed. 2nd ed. **1**: 198. J. B. Lippincott and Co. Philadelphia, Pa.
2. MITCHISON, N. A. & O. L. DUBE. 1955. Studies on the immunological response to foreign tumor transplants in the mouse. II. The relation between hemagglutinating antibody and graft resistance in the normal mouse and mice pretreated with tissue preparations. *J. Exptl. Med.* **102**: 179.
3. CHASE, M. W., 1955. The role of the formal elements of the blood in allergy and hypersensitivity. *J. Allergy.* **26**: 242.
4. RAMMELKAMP, C. H. Unpublished observations.
5. LAWRENCE, H. S. Unpublished observations.
6. KOURILSKY, R., G. DE CROIX & P. GANTER. 1952. Études sur l'allergie tuberculinique: la transmission de l'allergie. *Rev. immunol.* **16**: 333.
7. FREUND, J. & M. M. LIPTON. 1955. Experimental allergic encephalomyelitis after the excision of the injection site of antigen-adjuvant emulsion. *J. Immunol.* **75**: 454.

STUDIES ON THE ROLE OF ANTIBODIES IN THE FAILURE OF HOMOGRAFTS*

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The purpose of our study has been to ascertain the validity of the immunological concept in the failure of homografts. We shall not refer here to the well-known researches of Medawar, Converse, Billingham, Conway, Dempster, Simonsen, Taylor, Lehrfeld, and others, but confine ourselves to a description of our own work.

Our researches on skin homografts began in 1953 and have assumed 3 phases:

(1) We first sought evidence in favor of the immunological concept. With this end in view, we confirmed the second-set phenomenon and conducted other experiments of an immunological nature.

(2) We then attempted to formulate tests proving cutaneous sensitization of the receptor toward the donor. At the same time we attempted to find circulating antibodies.

(3) Finally, in trying to transfer passively the hypersensitivity to the graft by means of the cells of receptor animals, we attempted to demonstrate the presence of intracellular antibodies.

Methods

We used rabbits and guinea pigs as the subjects of our experiments, as follows:

Rabbits. Forty-eight rabbits were employed. These weighed from 1500 to 2500 grams each, and were usually males of various strains.

Guinea pigs. We used 434 guinea pigs, weighing from 400 to 600 grams each, usually males, and of the agouti or the tricolor strains, but not of pure strains.

Techniques

Grafts. The surgical technique used (FIGURE 1) was similar in both rabbits and guinea pigs. We give here only its main points:¹

(1) The skin transplant is generally square in shape, 20 to 40 mm. in size, of full-thickness abdominal skin cut with a scalpel (suprapannicular graft).

(2) The bed of the graft is prepared by complete excision of the skin and the subcutaneous tissues down to the muscle (subpannicular bed). The usual receptor site is the lateral thoracic region, the underlying skeletal framework permitting the best immobilization of the graft.

(3) Fixation of the graft is done by means of 4 sutures at the angles including the subjacent muscle; 1 or 2 sutures are then sufficient for each of the sides of the graft.

(4) Immobilization of the graft, the essential point, is obtained by means of a rolled piece of gauze fixed by sutures that are anchored to the skin at some

* The work described in this paper was supported by the Association Claude Bernard des Hôpitaux de Paris, and the Centre National de la Recherche Scientifique, Paris, France.

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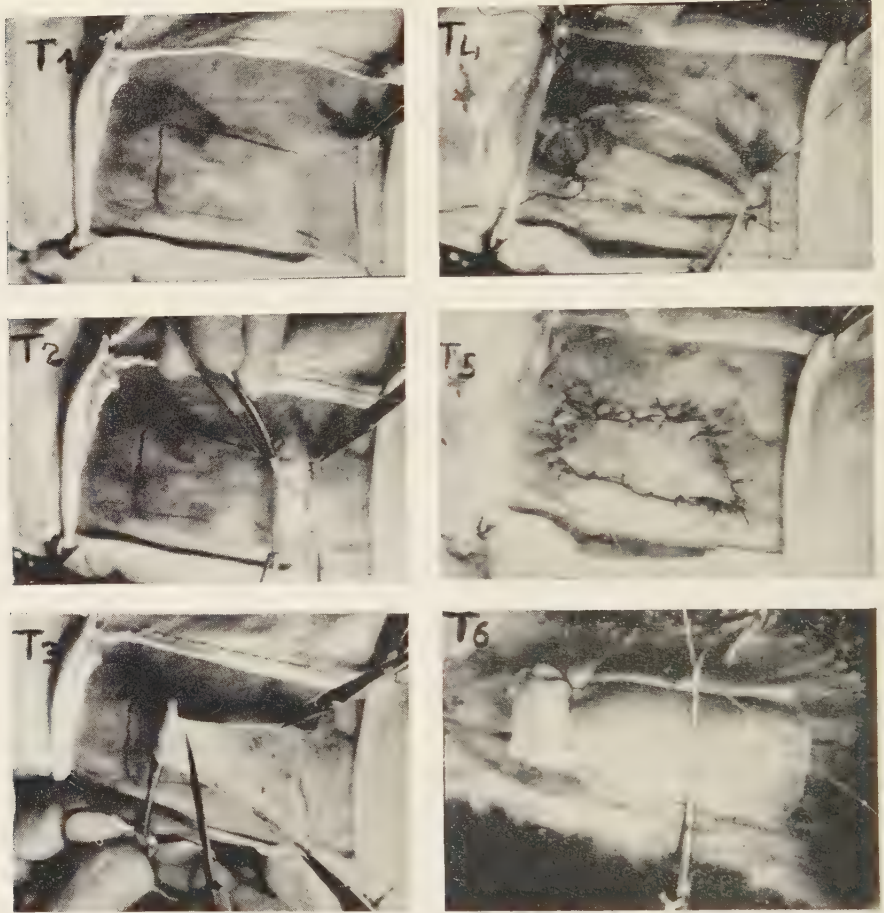


FIGURE 1. The technique used for grafts in rabbits. T1, T2, and T3 show the removal of a suprapannicular graft. T4, T5, and T6 show how a graft is placed and maintained on the subpannicular bed. The identical technique was used in the experiments with guinea pigs.

distance from the graft, the sutures being passed through the skin in the subjacent muscle.

Observations of the Grafts

Usually observations were made by palpation and by naked eye, noting the general aspect of the graft, its color, its temperature, its consistency, and its state of fixation at the periphery and in the deeper tissues. Adequate information was thus obtained concerning the vitality and the vascularization of the graft. Observations were made between the 5th and 12th days, rarely sooner and only exceptionally later, and usually on the 5th, 7th, 9th, 10th, 11th, and 12th days. In some cases daily observations were made. According to the

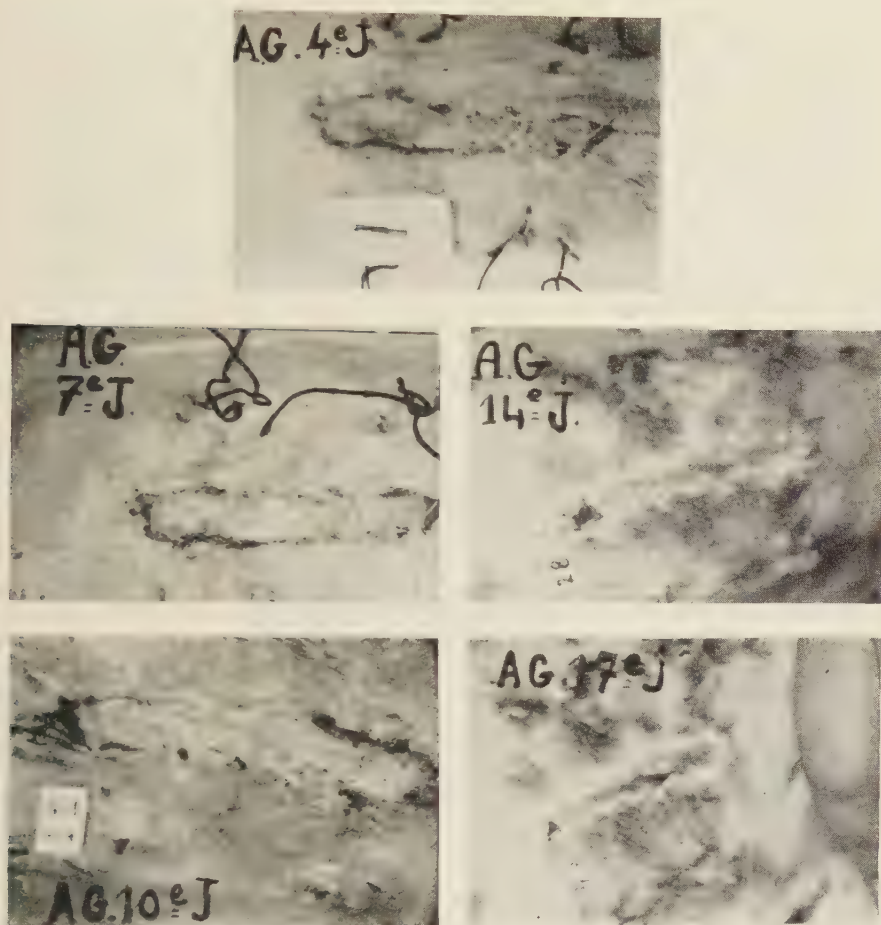


FIGURE 2. The evolution usually observed in autografts of rabbits. Shown are the aspects of the same graft on days 4, 7, 10, 14, and 17. On day 14, the macroscopic aspect of the graft is nearly that of normal skin. Nearly the same aspect was found in the experiments with guinea pigs.

objective findings, the condition of the graft was described as follows: (1) alive and of (a) good aspect, (b) average aspect (both of these aspects being similar to those of the autografts shown in FIGURE 2), and (c) mediocre aspect; (2) dead and, in appearance, (d) livid, (e) clear brown, (f) necrotic, dry, and black; (3) sometimes of an aspect intermediate between the aspects previously described. Between the 7th and the 12th days the appearance of the graft is characterized by a total desquamation of the epithelium, leaving an oozing dermis. This aspect usually occurs 24 to 48 hours before necrosis of the graft.

Since we waited for the beginning of the necrosis before speaking of the "dead grafts," survival times are usually 48 hours longer than those described by Taylor and Lehrfeld,² who used stereomicroscopic observations of the grafts.

Seventy-three biopsies were done to check the gross appearance of the grafts by histological examination or to investigate certain histological aspects.

EXPERIMENTS IN FAVOR OF THE IMMUNOLOGICAL CONCEPT³

(1) Second-set phenomenon (21 rabbits and 20 guinea pigs were used for these experiments).

Confirmation of Medawar's⁴ research on rabbits. Instead of being rejected in about 12 days in a manner characteristic of a first-set graft (FIGURE 3), we observed that a second graft from the same donor to the same receptor is rejected in about 8 days (FIGURE 4). Confirming Medawar's observations, but

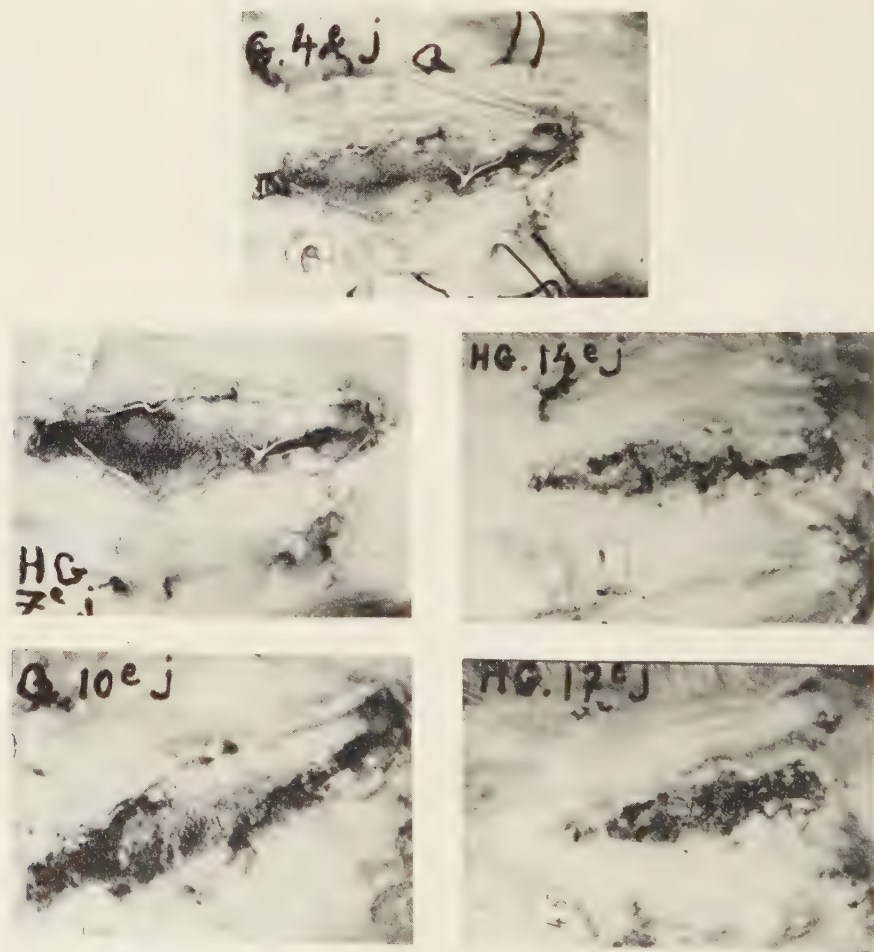


FIGURE 3. The evolution usually observed in homografts of rabbits. The aspect of the same graft of black skin on days 4, 7, 10, 14, and 17. On day 10 there was some necrosis at the 2 ends of the graft and along its lower edge. All of the graft was dead on day 14. The evolution of grafts in guinea pigs is nearly the same.

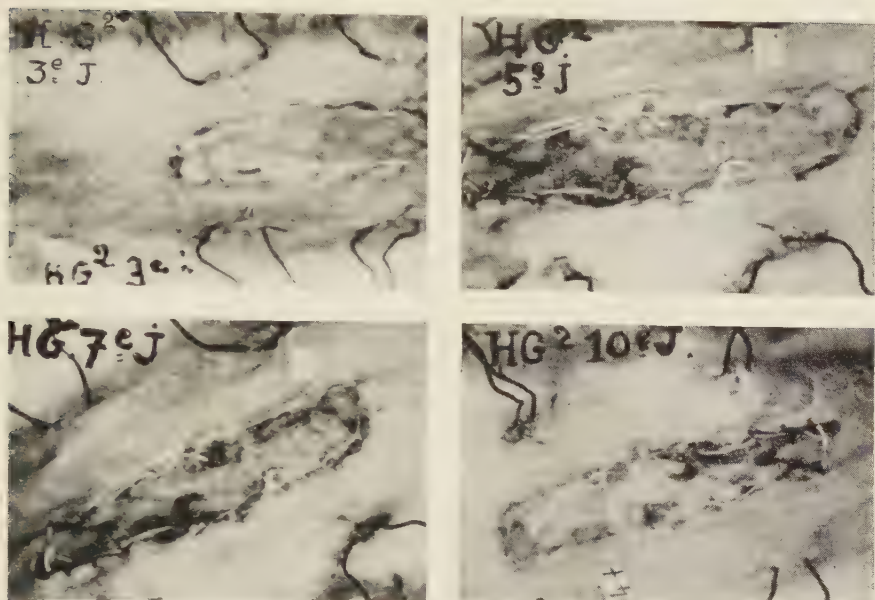


FIGURE 4. The evolution usually observed in second-set homografts in rabbits. Shown is the aspect of such a graft on days 3, 5, 7, and 10. All of the graft was dead on day 10. The evolution of second-set homografts is nearly the same in guinea pigs.

disproving those of Conway,⁵ we observed vascularization of the homografts in both first- and second-set homografts, from the same donor to the same receptor, in the form of parallel new capillaries that proliferate from the muscular bed of the transplant.

Other observations included the following:

In rabbits we observed a congestive reaction of the receptor's skin around the graft toward the second day, but only in the case of the second homograft from the same donor to the same receptor without any infection.

In rabbits, a third homograft from the same donor to the same receptor was rejected somewhat more rapidly than the second homograft (in about 6 days).

In guinea pigs, the second-set phenomenon, which we have confirmed, constitutes the most important argument in favor of the immunological origin of the failure of skin homografts. There has been some question in recent experimental work as to whether or not a second wound heals more rapidly than a first wound. We attempted, therefore, to obtain further proof of the immunological origin of the failure of homografts.

(2) Skin grafts to animals actively sensitized by the skin of the donors (16 rabbits were used in these experiments).

Technique. About 2 grams of total skin of the future donor were finely cut up with scissors and ground in a mortar with 5 ml. of physiological saline and emulsified with 15 ml. of adjuvant substance of the type described by

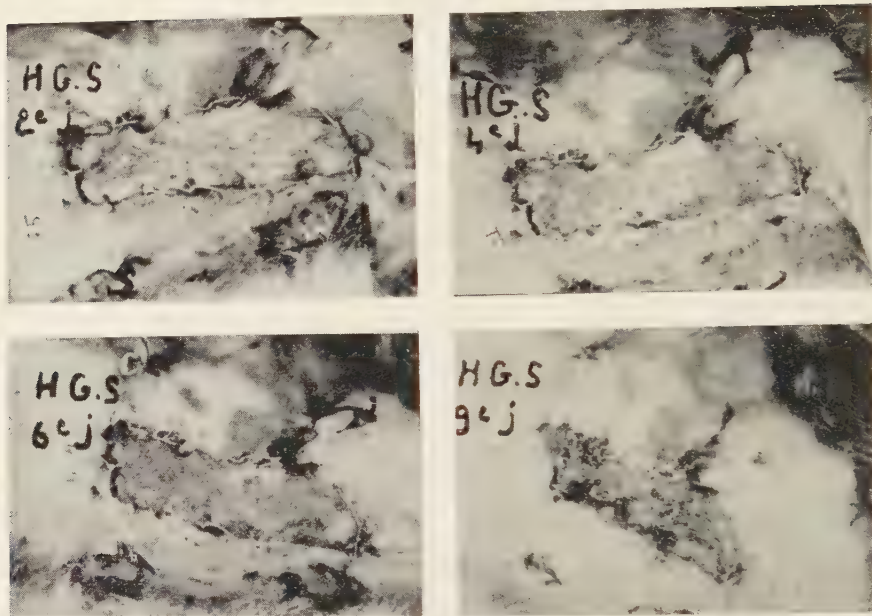


FIGURE 5. The evolution observed in first-set homografts in rabbits that had been sensitized previously to a homogenate of the skin of the donor. The graft, still alive on day 6, was dead on day 9.

Freund.⁶ Two to 4 injections of this mixture were administered at intervals of 5 days to the future receptor. The latter received a graft from the same donor one week after the last injection.

Results. The evolution of the graft was comparable to that of a second-set homograft from the same donor to the same receptor (FIGURE 5). It was rejected around the ninth day.

Two facts may be noted in these experiments:

(a) Four rabbits were injected with an emulsion of ground skin of the future donor, together with adjuvant substances of the same composition as the preceding ones, except that they contained no bacteria. We thought that this mixture could play the role of the hapten, nonantigenic *in vivo*, but capable of fixing antigraft antibodies elaborated by the receptor. We hoped to protect the graft in this manner and prolong its length of survival. We found that the grafts made on these animals evolved exactly as if the adjuvant substances had contained mycobacteria, and they were rejected in about 9 days. Contrary to our intention, the receptors were thus sensitized to the graft. Thus, the mycobacteria do not appear to be indispensable to elicit antigraft sensitization.

(b) In the 2 preceding groups of animals sensitized to the skin of the future donor and having received a homograft of skin from the same donor, we were surprised to note, 2 months later and quite by accident, the formation of a dermatosis.⁷ The dermatosis was characterized (FIGURE 6) clinically by a

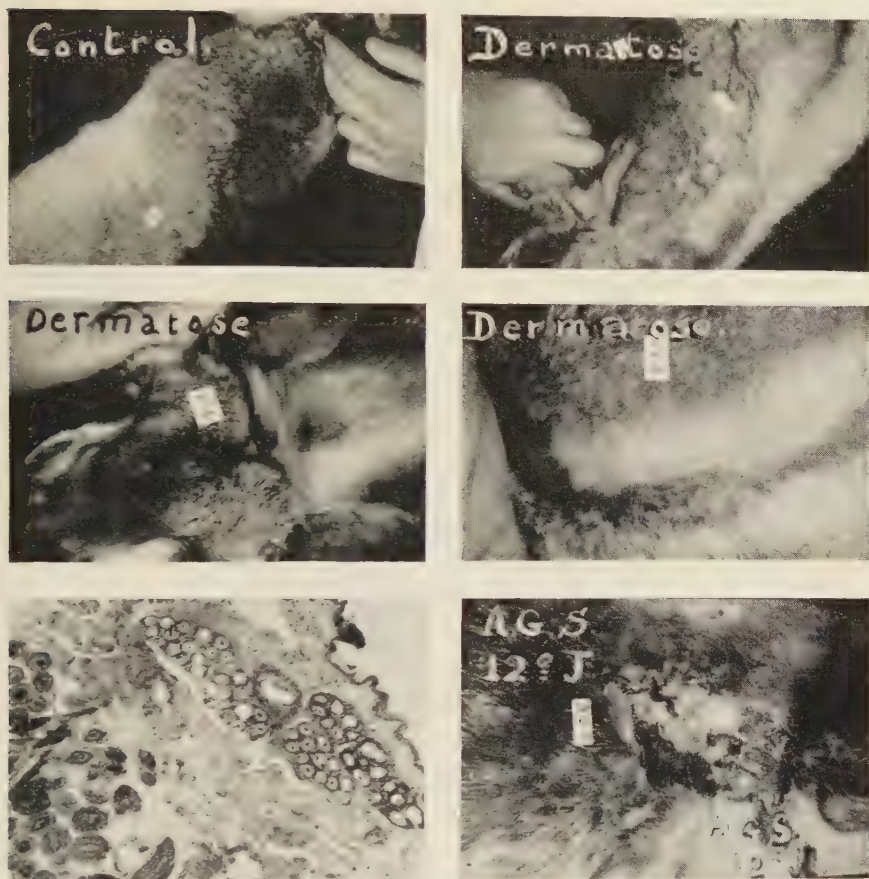


FIGURE 6. The dermatosis observed on rabbits that had been sensitized to a homogenate of the skin of the donor and subsequently grafted with the skin of the same donor.

Upper left, control. Upper right and center, macroscopic view of the dermatosis (note areas of alopecia, often perivascular). Lower left, microscopic view of the dermatosis (note the thinning of the epithelium and the necrosis of the superficial hair follicles). Lower right, the aspect of a 12 day old autograft in 1 of these rabbits. The graft is dead and has the appearance of a homograft, except for a small part along the middle of the upper edge.

disseminated alopecia, often distributed around the veins with thinning of the skin. Histologically, desquamation and thinning of the epithelium with necrosis of the superficial hair follicles were noted. Concomitantly to the necrosis of the superficial hair follicles, new hair follicles were formed more deeply in the skin. The donor animals for skin which cohabitated with the recipient animals did not show any phenomenon of this type. Rabbits that had received adjuvant substances mixed with extract of kidney and testicle did not present such cutaneous disturbances.

We performed autografts in 6 animals presenting this pathological cutaneous reaction, and in 4 cases the evolution was comparable to that of a homograft

up to the 12th day. It was later noted that a small portion of the graft which had survived, two thirds or three fourths of the graft having become necrosed, had an evolution characteristic to that of an autograft.

All of these phenomena seem compatible with the formation of antibodies against rabbit skin. These antibodies seem to have been the cause of both the dermatoses and necroses of a portion of the autograft.⁵ No other hypothesis seems to be compatible with the observed facts, but we are still unable to adduce direct proof.

(3) Grafts of skin on animals passively sensitized to the skin of the donor: action of serum heteroantibodies. (These experiments on 20 guinea pigs were undertaken to explore the mechanism of the phenomena previously described.)

Technique. Rabbit-immune sera are prepared against the skin of the future donor guinea pig or against the skin of a future nondonor guinea pig. Into the grafted guinea pigs (homografted, autografted, or both), is injected 1 of

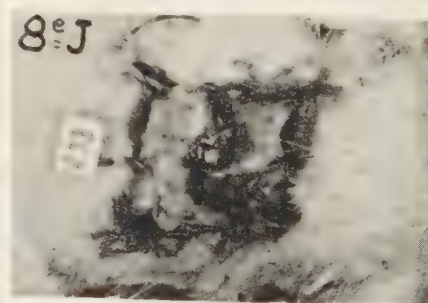
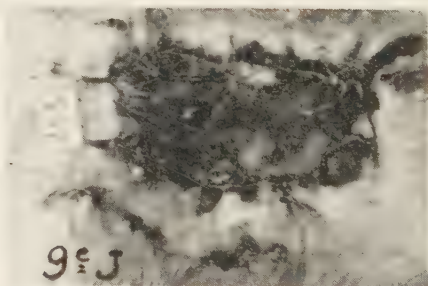
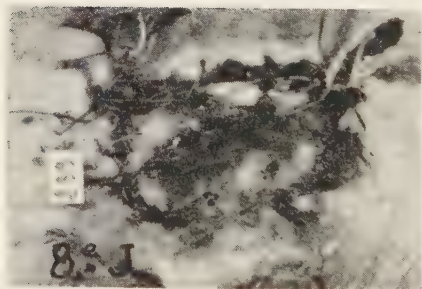
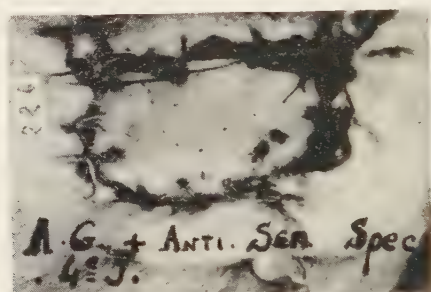


FIGURE 7. Aspects of an autograft (3 upper photographs) on days 4, 8, and 9, and of a homograft (lower pair) on days 4 and 8, both on guinea pigs that had received rabbit antisera against the donor's skin.

the preceding sera (or a serum of a nonsensitive, normal rabbit) 3 days after the graft.

Results. (1) Homografts in guinea pigs that had received normal rabbit sera died in 10 to 12 days. (2) Homografts in guinea pigs which had received donor's antiskin sera died in 6 to 8 days (in 8 cases out of 9, FIGURE 7). (3) Homografts in guinea pigs that had received antiskin sera of nondonor guinea pigs died in 8 to 10 days in all cases. (4) Three autografts in guinea pigs that had received rabbit antisera, specific or nonspecific of the graft, died in 8 to 10 days (FIGURE 7). These animals had received on the ipsolateral side of the trunk a homograft that also died in 8 to 10 days. In 3 other autografts, serum having been injected 15 days after the grafting, the grafts were not affected.

One can conclude that anti-guinea pig skin rabbit sera shortened the survival time of homografts, especially if they were specific to the skin of the donor. Under certain conditions even the autograft may be rejected as a result of the action of the serum.

Conclusions. For the following reasons, it seems highly probable that the second-set phenomenon is a purely immunological one: (1) It is reproducible by active sensitization of the future receptor to the future donor without grafting, simply by injecting ground skin mixed with adjuvants. (2) It is reproducible by passive sensitization of the future receptor to the future donor by means of serum heteroantibody.

One may add that the antibodies in question are very probably anti-individual antibodies, but that antiorgan antibodies may play an equal role, since the evolution of skin autografts is influenced in animals affected with an allergic dermatosis and since rapid rejection of the skin homograft, and even of a skin autograft, occurs under the influence of antiskin heteroantibodies of the homografted species.

SEARCH FOR PROOF OF A SENSITIZATION OF THE RECEPTOR TOWARD THE DONOR SKIN

All the preceding arguments and those of numerous authors who have worked on this problem are thus in favor of the concept according to which the death of the homograft is due to the elaboration by the host of antibodies against the graft. The existence of these antibodies remains to be demonstrated; in this second series of experiments we have attempted to accomplish this.

Any experiment of an immunological nature presupposes an antigen. We have prepared the antigen with the skin of the donor in the following manner:

One gram of skin is very finely cut up and placed in a mortar with 2 to 3 gm. of silicious sand; the skin is ground for a long time, progressively adding 4 gm. of normal saline solution. After centrifuging for a period of 40 minutes, the supernatant fluid is filtered. This aqueous extract is utilized as the antigen to be diluted to the need of each individual experiment.

Search for Cutaneous Sensitization

Three rabbits and 2 guinea pigs that had received 2 homografts from the same donor were injected intradermally with 0.2 ml. of the aqueous extract,

pure or diluted 1:20, prepared from the donor. They were also injected with similar extracts from a nondonor animal as a control. The sites of the injections were examined every day for 5 days. We noted an occasional inflammatory reaction appearing on the first day and lasting 3 to 4 days. It was no more particularly marked in the sites of injections of the donor's aqueous extract than in those of injections of the aqueous extract prepared from the nondonor animal.

Search for Circulating Antibodies

Ring test. The serum of 8 rabbits that had received 2 homografts from the same donor was put in contact with an aqueous extract (diluted 1:100) of skin of the same donor. We failed to observe the formation of an interfacial precipitation.

*General anaphylaxis.*⁹ Nine guinea pigs that had received 2 homografts from the same donor received an intravenous injection of 1 ml. of the extract of the donor's skin. Nine nongrafted control guinea pigs received the same injection. The control animals failed to show any disturbance. Two grafted animals presented a slight disturbance with expiratory bradypnea.

Local passive anaphylaxis (Ovary test¹⁰). Young guinea pigs with white bellies were injected intradermically with sera from 4 guinea pigs that had previously been homografted, and with the control sera of 4 normal guinea pigs. Six hours later these white-bellied guinea pigs were injected with a mixture of Evan's Blue Dye and an extract of donor's skin. We observed no passage of Evan's Blue at the site of intradermic injection of serum from the grafted guinea pigs.

Conclusions. (1) We have been unable to show evidence of a cutaneous sensitization of the host to an aqueous extract of skin of the donor, as the observed reactions were not specific. (2) We have been unable to demonstrate, with the methods which have been utilized, the existence of circulating serum antibodies specific for the donor's skin.

These results, which confirm those of other workers, seem to point to the fact that antibodies responsible for the failure of homografts do not circulate in the serum, but may be intracellular. The observation of disturbances with bradypnea that might be interpreted as slight anaphylactic shock in these 2 guinea pigs that had received an intravenous injection with skin extract from their skin donor do not contradict this view, as the role of fixed antibodies in the production of anaphylactic shock is well known.

These experiments led us to the necessity of undertaking a series of experiments to detect the presence of intracellular antibodies.¹¹

SEARCH FOR INTRACELLULAR ANTIBODIES RESPONSIBLE FOR HYPERSENSITIVITY TOWARD THE GRAFT

Passive transfer of hypersensitivity to the graft by means of lymphocytes and macrophages. In this series of experiments, 375 guinea pigs were used. We attempted to transfer passively to a guinea pig hypersensitivity toward the

donor by transferring to him the macrophage and lymphocyte cells of guinea pigs that had already been homografted from the same donor. The techniques we employed were similar to those employed by Landsteiner and Chase¹² for the transfer of hypersensitivity to picryl chloride, and by Chase¹³ for hypersensitivity to tuberculin.

The procedure for this experiment follows (FIGURE 8). On day 0 the skin from a donor guinea pig A was grafted to 8 guinea pigs (first hosts). On day 15, skin was grafted from the same donor, guinea pig A, to 4 other previously

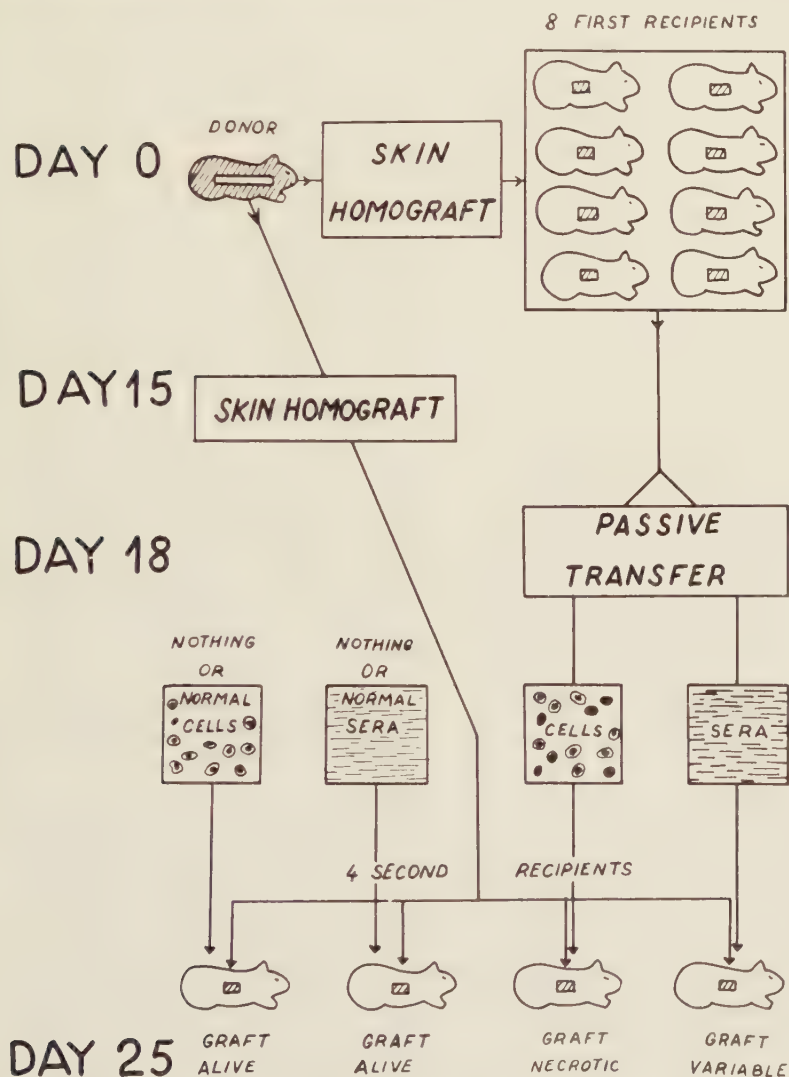


FIGURE 8. Diagram of the experiments of passive transfer of hypersensitivity toward the graft.

nonoperated guinea pigs (second hosts). On day 18, serum, macrophages, and lymphocytes were obtained from the first 8 guinea pigs. Cells were then injected into one of the 4 second-host guinea pigs. Serum was injected into another of the 4 second-host guinea pigs. The other 2 second-host guinea pigs received either no injection, or else the cells and serum of 8 nonoperated guinea pigs. On days 20, 22, 24, 25, 26, and 27 (on the fifth, seventh, ninth, tenth, eleventh, and twelfth postoperative days) the 4 grafts were examined, thus completing the experiment.

Technique

The grafts were applied following the previously described technique.

The sera were obtained by cardiac puncture immediately before sacrificing the animal in order to collect the cells.

The cells included macrophages and lymphocytes.

The macrophages were obtained from peritoneal exudates that were formed after the intraperitoneal injection of paraffin oil 3 days previously. The peritoneum was opened and washed 3 times with 20 ml. of Hank's solution containing 0.1 of serum of a normal guinea pig. This liquid was decanted and centrifuged, and the residue washed and recentrifuged.

The lymphocytes were collected by dissociation of ganglia in the spleen of the first guinea pig host by means of fine needles. The cells were filtered on sterile gauze, centrifuged, washed, and recentrifuged.

The injection of these cells in to one of the second guinea pig hosts was usually intraperitoneal. The cells, macrophages, lymphocytes, or a mixture of both cells were suspended in 20 ml. of Hanks' solution containing 0.1 of normal guinea pig serum.

In some cases the injections were made intravenously. In such cases the macrophages were resuspended in 1 to 1.5 ml. of Hanks' solution, and 6 second guinea pig hosts were utilized: 2 of them received the cells of the first guinea pig hosts, 1 receiving the macrophages intravenously, the other the lymphocytes intraperitoneally.

Timing

In order to determine the point at which the cells had accrued the maximal titer of antibodies and in order to time their action, the cell transfer was performed 10, 15, or 21 days after the graft to the first guinea pig host, and 1, 3, or 5 days after the graft to the second guinea pig host. Usually the delay was 3 days in the case of the intraperitoneal injection of the cells, and 5 days in the few cases of intravenous injection.

Photographs were made frequently during the observation of the graft on the second guinea pig hosts.

Biopsies were done only in those cases in which the gross aspect was doubtful. When the evolution of the graft was typical, we preferred not to disturb it.

Results

We undertook 32 experiments of the passive-transfer type. In 17 cases the experiment was not carried to its conclusion (in 8 cases the donor died before

the second series of homografts; in 3 cases the second series could not be done; and in 6 cases the animal which received the cells died before the seventh day after the graft).

In 15 cases the animal receiving the cells and 1 control animal lived at least 8 days after the graft, and the experiment could be considered valid. Of these 15 experiments we must set aside 1 in which the second grafts lived longer than 15 days.

Of the remaining 14 experiments, 18 second guinea pig hosts received the cells of the corresponding first hosts, 10 second hosts received the sera of the first hosts, and 27 second hosts received the normal cells, normal sera, or nothing.

We were not always able to determine the day of a graft's rejection. Therefore, the mean survival time of the grafts' rejection can be determined only approximately. These survival times were 8 to 9 days in the 18 animals that had received "specific cells"; 9 to 10 days in those that had received "specific sera" (10 animals in all); and 10 to 12 days in the 27 control animals.

On the other hand, we were able to compare the condition of the grafts of the second guinea pig hosts of each series, especially between the seventh and eleventh days (FIGURES 9, 10, and 11).

Under these conditions, and in the 14 experiments to which we refer, if we consider the aspect of the grafts of the animals that had received the cells of the first guinea pig hosts, we observed the following facts:

In 12 of the 18 guinea pigs that had received "specific cells," the "specific-cells" graft presented an aspect distinctly worse than that of the others. In 5

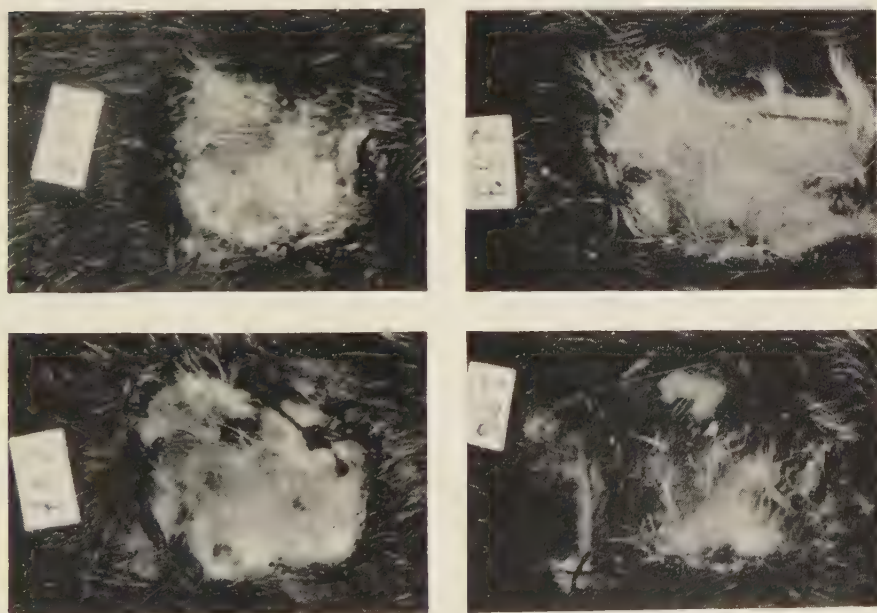


FIGURE 9. Experiment No. 18 of passive transfer of hypersensitivity toward the graft in guinea pigs. Shown are the aspects of the homografts on day 7. Upper pair, control. Lower left, specific-sera treated animal. Lower right, specific-cell treated animal.

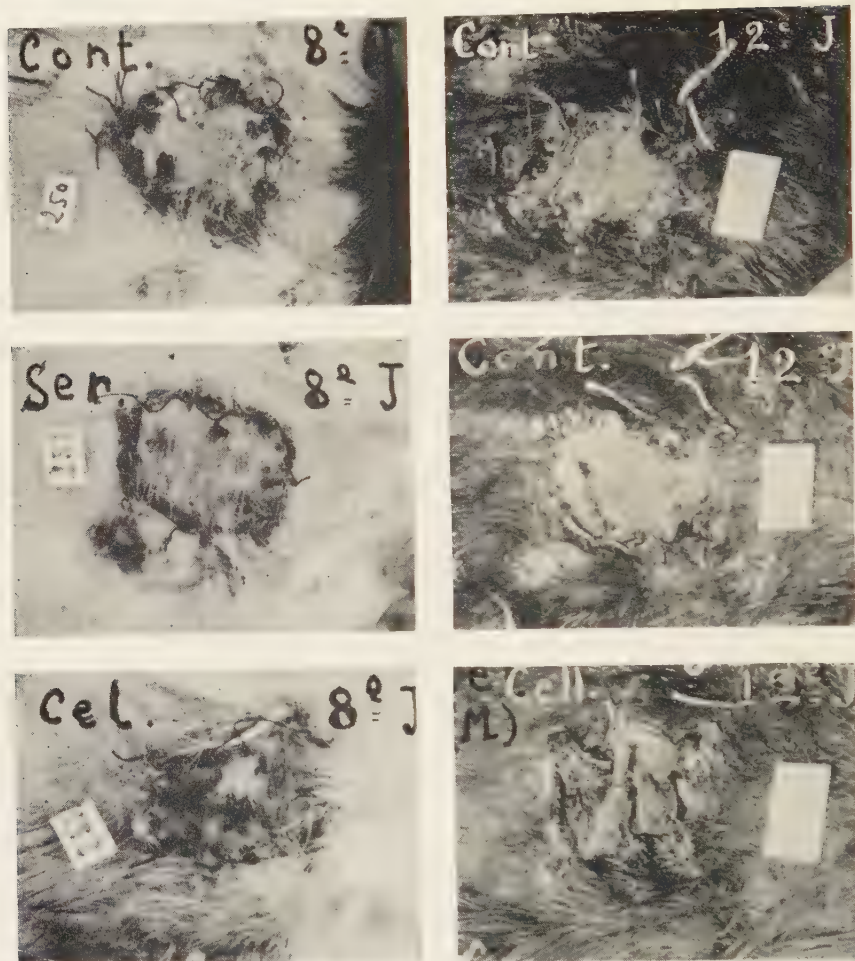


FIGURE 10. Two experiments, using guinea pigs, of passive transfer of hypersensitivity toward the graft. At the left is experiment No. 3, showing the aspects of the homografts on day 8. At the right is experiment No. 27, showing the aspects of the homografts on day 12. Upper pair, controls. Middle left, specific-sera treated animal. Middle right, control. Lower pair, specific-cell treated animals.

cases this difference was spectacular, the "specific-cells" graft being necrotic while the other grafts were alive. In 5 cases, not as spectacular, the difference was very clear. In 2 cases the difference was present, but it was less marked. On 2 occasions the aspect of this graft was more satisfactory than that of the "specific-serum" graft, but was less satisfactory than the aspect of the control grafts. On 2 occasions this was identical to the control grafts. On 2 occasions it was more satisfactory than one of the control grafts.

If we consider the appearance of the graft in the animals that have received the sera of the first guinea pig hosts, we observe the following facts:

On 3 occasions this "specific-serum" graft showed an aspect that appeared

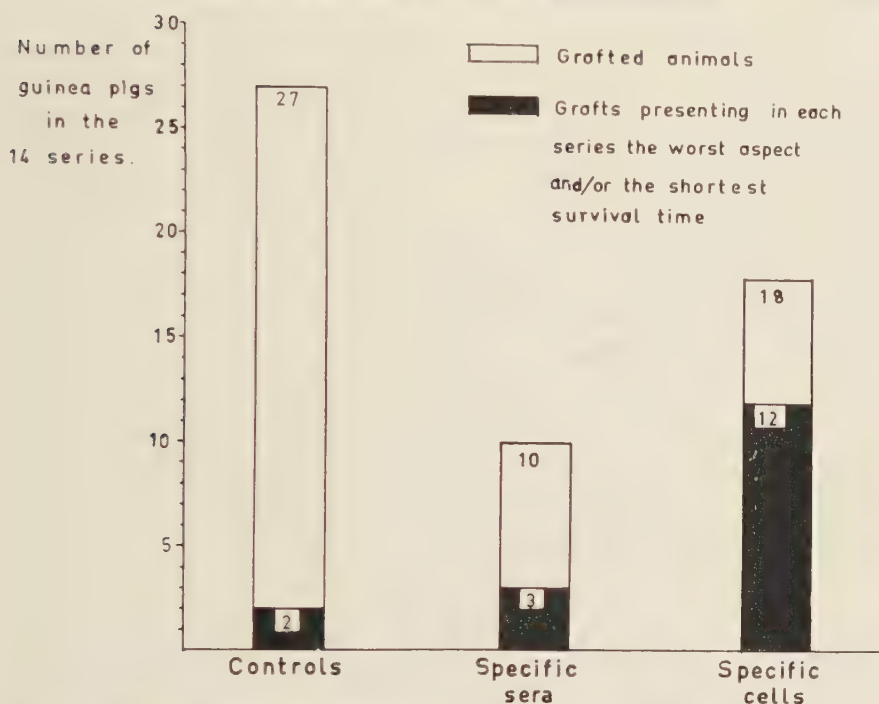


FIGURE 11. Graph showing the numerical data concerning the experiments on the passive transfer of hypersensitivity toward the graft.

to be in worse condition than the others. On 2 occasions this difference was spectacular, and on 1 occasion it was distinct.

In two cases the appearance of the aspect of the "specific-serum" graft was more satisfactory than that of the "specific-cells" graft, but less satisfactory than that of the control graft.

In 3 cases the appearance of the grafts was more satisfactory than that of all the other grafts, and in 1 case the appearance was comparable to that of one of the controls.

Five animals that had received the "specific serum" died prematurely.

In considering the appearance of the grafts of the 27 control animals that had received nothing or that had received cells or serum from normal guinea pigs, on only 2 occasions did the control grafts show a worse appearance than on animals that had received "specific cells" or "specific serum."

There are a few other points worthy of note:

(1) In the 3 experiments in which the serum seemed to have had a definite influence on the graft survival, the cell transfer seemed to have had a less definite influence or no influence at all. In 2 experiments in which this "anti-graft" action seemed about equal, it was less intense than when it was definitely noted either following cell transfer or following serum injection. These facts suggest that antibodies that are habitually intracellular can, under certain circumstances, be liberated in the serum.

(2) In 4 experiments we attempted to compare the relative efficiency of macrophages and lymphocytes in transferring sensitivity. We are unable, however, to note any difference. On the contrary, there appears to be a certain parallelism, since if 1 kind of cell (lymphocytes, for instance) succeeds in transferring hypersensitivity to the graft, then the other kind (macrophages) coming from the same animals succeeds also. Similarly, if one does not succeed, neither does the other.

(3) We were unable to find any notable differences between the transfers performed 10, 15, and 21 days after the grafts to the first guinea pig hosts and the cases in which the transfers were done 1, 3, and 5 days after the grafts to the second guinea pig hosts.

(4) When the gross appearance of the grafts did not permit a sufficiently precise evaluation of the state of the graft, biopsies were done. The biopsies showed a particular histological picture in the "specific-cells" graft: that is, a thinner epithelium without mitosis in the basal layer; dilatation of the capillaries, which were filled with leukocytes; and presence of some eosinophiles in the dermis. We do not know the histological picture of the "specific-cells" graft in the cases where the transfer of hypersensitivity was grossly striking since, in order not to disturb the graft, we did not undertake a biopsy.

Conclusions

It is thus possible to transmit passively acquired hypersensitivity to the homograft by injecting into a recently homografted animal cells from another animal that had earlier received homografts from the same donor.

Under these conditions the evolution of the graft is often similar to that which is characteristic of the second-set graft.

It thus appears that, without being absolutely constant, the influence of the evolution of the homograft of the cells from the first guinea pig hosts is a definite one.

The influence of the serum of the first guinea pig host is far more irregular and does not, to date, permit clear conclusions.

DISCUSSION AND GENERAL CONCLUSIONS

Such are the facts that we have observed. What light do they bring to the problem of the role of antibodies in the failure of homografts? The value of the experiments described in this paper is obviously dependent upon the value of the methods employed.

Value of the Methods Utilized

Our habitual method of examination of the grafts was a gross one. The 73 biopsies that we performed were done only to obtain precise information on particular points. We were unable to utilize the stereomicroscopic technique described by Taylor and Lehrfeld.² By careful surgical technique and gross examination of the grafts it was possible to tabulate our results, which were found to be reproducible. There remain 2 variable points: first, the variable vitality of the grafts coming from different animals and, second, the variable

susceptibility of different receptors toward the same donor. We ensured ourselves against these possible variations, which appeared to us to be infrequent, by the large number of experiments and by proceeding always in a comparable manner, grafting the skin of the same donor in several receptors. This was particularly true in the experiments of passive transfer in which we utilized 4 to 8 hosts for the same donor. Under these conditions, we feel justified in considering the results as valid.

Significance of the Results

It is possible artificially to reproduce a phenomenon with all the appearances of a second-set phenomenon by purely immunological means that produce a sensitization of the receptor to the donor.

We have achieved this sensitization in 3 ways: first, actively, by injecting into the future host the ground skin of the future donor mixed with adjuvants; second, passively, by injecting into the host an antigraft serum (the effect is certain when we deal with a heterologous antiserum—it is less certain when we deal with a homologous serum of an animal that previously had received a homograft from the same donor); and third, passively, by injection of homologous cells (lymphocytes and macrophages) of animals that had previously received a homograft from the future donor.

From these facts the following conclusions may be drawn:

(1) It is logical to attribute to the second-set phenomenon a purely immunological significance caused by substances which have the properties of antibodies. We must note, in passing, that the immunological origin of the second-set phenomenon does not automatically imply that the death of the first graft is due to purely immunological phenomena. Nevertheless, there are certain arguments⁸ in favor of this theory, particularly the survival of homografts in agammaglobulinemic patients.

(2) Intracellular antibodylike substances, or some closely related mechanism, are the only ones the role of which seems firmly established in the accelerated rejection of the homograft in the second-set phenomena. This is in accord with the following 2 facts:

(a) A homograft can survive in a chamber that does not allow the penetration of cells. This chamber can be either natural—the anterior chamber of the eye (Medawar¹⁴)—or artificial—the porous chambers of Algire¹⁵ and Woodruff.¹⁶

(b) The graft is not rejected before being well vascularized, which happens on about the fifth or sixth day, even in third- or fourth-set homografts of the same donor to the same host.

(3) The role of the serum as a carrier of the presumptive antibodies is not clearly understandable and even appears doubtful. It appears that the serum has, nevertheless, some role in the rejection of the homograft for the following reasons: homografts can survive in agammaglobulinemic patients (Good¹⁷). Serum from homografted animals has some degree of cytotoxicity toward the epidermal cells of the donor's skin (Billingham¹⁸).

Serum from homografted animals appears to have, in a very few cases, an effect in passive-transfer experiments such as was described in the third part

of this paper. But as was reported in the second part of this paper, we have as yet been unable to find any circulating serum antibodies in homografted animals.

Strong graft-specific heterosera have a striking effect on homografts, as was stated in the first part of this report. One must keep in mind, however, the probable multiplicity of such heteroantibodies directed against numerous constituents of the graft.¹⁹ Some of these are probably individual-specific, others species-specific, others organ-specific. Furthermore, all of these heteroantibodies must contribute to accelerating the death of the graft.

(4) Finally, our impression and our working hypothesis is that antibodies involved in the rejection of the skin homografts are intracellular. They are neither very strong nor very abundant, however, and, under certain still unknown conditions, they may be released in the serum in rather small amounts.

References

1. MAURER, P. 1954. Contribution à l'étude des homogreffes cutanées. Thèse Méd. Paris, France.
2. TAYLOR, A. C. & J. W. LEHRFELD. 1955. Definition of survival time of homografts. *Ann. N. Y. Acad. Sci.* **59**(3): 351-360.
3. MAURER, P. & G. VOISIN. 1954. Contribution à l'étude des homogreffes cutanées. *Mém. acad. chir.* **33** & **34**: 927.
4. MEDAWAR, P. B. 1945. A second study of the behaviour and fate of skin homografts in rabbits. *J. Anat.* **79**: 157-176.
5. CONWAY, H., D. JOSLIN & R. B. STARK. 1951-1953. Observations on the development of circulation in skin grafts. *Plast. reconstr. Surg.* **1-8**.
6. FREUND, J. & K. McDERMOTT. 1942. Sensitization to horse serum by means of adjuvants. *Proc. Soc. Exptl. Biol. Med.* **49**: 548-553.
7. VOISIN, G. & P. MAURER. 1955. Sur une dermatose expérimentale apparue chez des lapins traités par des extraits de peau homologue. *Sem. Hôp. Paris.* **31**: 1909-1912.
8. VOISIN, G. 1955. Les auto anticorps cytotoxiques. Étude critique, clinique et expérimentale. 30e Congrès français de médecine. Alger. **II**: 225-264. Masson, Paris, France.
9. VOISIN, G. & A. DELAUNAY. 1955. Mise en évidence d'un état d'iso- ou d'autosensibilisation au moyen du choc anaphylactique. *Ann. inst. Pasteur.* **89**: 556-581.
10. OVARY, Z. & M. BRIOT. 1953. Nouvelle méthode de dosage de l'anticorps anaphylactique et son rapport avec l'azote de l'anticorps. *Ann. inst. Pasteur.* **84**: 620-671.
11. MAURER, P. & G. VOISIN. 1954. Les problèmes posés par l'étude biologique des greffes. *Rev. praticien.* **4**: 3097-3108.
12. LANDSTEINER, K. & M. W. CHASE. 1942. Experiments on transfer of cutaneous sensitivity to simple compounds. *Proc. Soc. Exptl. Biol. Med.* **49**: 688-690.
13. CHASE, M. W. 1945. The cellular transfer of cutaneous sensitivity to tuberculin. *Proc. Soc. Exptl. Biol. Med.* **59**: 134-135.
14. MEDAWAR, P. B. 1946. The fate of skin homografts transplanted to the brain, to subcutaneous tissue and to the anterior chamber of the eye. *Brit. J. Expt. Pathol.* **29**: 58.
15. ALGIRE, G. H. 1957. Studies on tissue homotransplantation in mice, using diffusion-chamber methods. *Ann. N. Y. Acad. Sci.* **64**(5): 1009.
16. WOODRUFF, M. F. 1957. Cellular and humoral factors in the immunity to skin homografts: experiments with a porous membrane. *Ann. N. Y. Acad. Sci.* **64**(5): 1014.
17. GOOD, R. A. 1957. Transplantation studies in patients with agammaglobulinemia. *Ann. N. Y. Acad. Sci.* **64**(5): 882.
18. BILLINGHAM, R. 1957. Studies on epidermal cell suspensions, with particular reference to problems of transplantation immunity. *Ann. N. Y. Acad. Sci.* **64**(5): 799.
19. VOISIN, G. 1955. Nature des antigènes tissulaires. 5e Congrès européen d'hématologie. Colloque d'immuno hématologie. Fribourg-en-Brisgau. In press.

Discussion of the Paper

BYRON H. WAKSMAN (*Department of Bacteriology and Immunology, Harvard Medical School, and Neurology Service, Massachusetts General Hospital, Boston,*

Mass.): The excellent experiments of Voisin and Maurer have involved the use of several procedures that are traditional in experimental pathology. These investigators find that injection of heteroantibody against skin in the guinea pig causes disease, manifested by graft rejection, in skin handicapped by the grafting procedure. This disease must be compared with the many well-known diseases produced with cytotoxic antisera, such as "Masugi" nephritis and experimental hemolytic anemia. Their finding is in agreement with the observations reported in this monograph by Algire on the effects of heteroantisera on grafts. That a handicap to survival of skin is created by the grafting procedure is suggested by Woodruff's finding that, in incompletely tolerant animals, a first graft survives even while a later graft from the same donor is breaking down. I suggest that the first graft had recovered from this difficulty before the appearance of weak immunity sufficient to destroy a fresh graft. The same point is further illustrated in Voisin and Maurer's autoallergic rabbits, in whom skin death occurred only when the grafting handicap was superimposed on the sensitivity already present.

Voisin and Maurer's second finding is that either immune cells or serum antibody may cause homograft rejection in homologous passive transfer. The finding with cells confirms the earlier work of Mitchison¹ and of Billingham, Brent, and Medawar,² and is in agreement with the considerable evidence that an immunologic reaction of the delayed type is responsible for homograft rejection. The finding with serum is in conflict with a good deal of earlier work (see Algire's and Woodruff's reports elsewhere in these pages) and is incompatible with the evidence for delayed sensitivity. While the data presented by Voisin would make the serum antibody appear a much less effective agent than immune cells in causing graft rejection, it may well be that both mechanisms play a role. This possibility has already been discussed here at length (Lawrence, Woodruff) and is certainly suggested by the studies of agammaglobulinemia (Good, Porter). The conflict with earlier data may depend on the use of different experimental species (guinea pigs as compared to rabbits, rats, and mice).

The third technique employed in the experiments under discussion is the use of subcutaneous injections of adjuvants plus skin to produce immunity comparable to that obtained with an immunizing graft. The usual living graft provides essentially an intradermal immunization. Medawar³ could obtain similar immunization with intradermal injections of leukocytes, and Billingham, in this monograph, describes his achievement of the same results using intradermal epidermal cells, while the same inocula by other routes failed to immunize. This situation is exactly comparable to the production of delayed sensitivity with streptococci,⁴ pneumococci,⁵ or even serum proteins.⁶ In all these cases, intradermal injection of antigen gives delayed sensitivity, while injection by other routes does not. Similarly, in contact allergy, the usual sensitizing exposure is via the skin. Sensitivity can be produced by other routes of administration only with the use of adjuvants, as in the present experiments. It should not come as a surprise to us to find that the type of sensitivity produced by living foreign cells (the graft) appears to be "infectious"

allergy. The use of adjuvants may well permit the use of dead cells in immunization (sensitization), as it does in other experimental situations.

Voisin and Maurer's observation that a skin disease, presumably due to autoallergy involving epidermis, is present in rabbits given skin and adjuvants, is of great interest. This finding goes beyond the earlier observations of Hecht, Sulzberger, and Weil⁷ on autosensitization to skin with the use of staphylococcal toxin as adjuvant. It is not clear what human disease this experimental process resembles. It appears to be a new member of the class of experimental autoallergies, all produced by injection of homologous or even autologous tissue with adjuvants. All those studied thus far appear to be experimental models of well-recognized human diseases. All appear to be reactions of mesenchymal tissue, that is, tissue of mesodermal origin, to antigens in tissue of ectodermal, endodermal, or other special origin. Among them, one may mention the following: autoallergy involving central nervous system myelin (ectodermal) produces so-called "experimental allergic encephalomyelitis," a disease resembling postinfectious or postvaccinal encephalomyelitis in man.^{8,9} Sensitivity to peripheral nervous system myelin (ectodermal) produces an "experimental allergic neuritis" that resembles infectious polyneuritis or the Guillain-Barré syndrome.¹⁰ A disease resembling human phacoanaphylactic endophthalmitis can be produced with autosensitivity to lens (ectodermal).¹¹ A disease of the uveal tract resembling sympathetic ophthalmia is produced with sensitization to retinal tissue (ectodermal).^{12,13} Aspermatogenesis produced by an autoallergy involving the primary sex cells in the testis (sex cells) has been extensively investigated.^{14,15} Apparently the thyroid (endodermal) can also act as an autoantigen in the production of experimental disease.¹⁶ And now the epidermis, the ectodermal tissue par excellence, is found to behave in a similar manner.

While I have explained the existence of these autoallergies, which so clearly contradict Ehrlich's principle of horror autotoxicus, as mesodermal reactions to tissue of other embryonic origin, they may be explicable on another basis. Many of the tissues involved are separated from the circulation by physiological barriers of one sort or another, for example, the blood-brain barrier. In consequence, tolerance to antigens in these tissues may fail to develop in fetal life, and the individual can react to them immunologically in adult life. Tolerance may also fail to develop if these antigens have not yet appeared at the critical period of fetal life. As the antigens in the other cases cited are certainly not living cells, but proteins or protein-lipid complexes that have been purified to varying degrees, it is probable that Voisin and Maurer's skin disease can be produced with nonliving antigen. In several human diseases (sympathetic ophthalmia, the Vogt-Koyanagi syndrome, Harada's disease), there is simultaneous involvement of the uveal tract, the central nervous system and meninges, the middle ear, and the skin and hair.^{17,18} In some work on experimental autoallergic reactions, a suggestion of similar overlapping involvement of the eye and central nervous system has been found.^{19,20} It becomes, then, of the greatest interest to know if Voisin has observed any disease of the eye or nervous system in his rabbits with skin disease.

References

1. MITCHISON, N. A. 1953. Passive transfer of transplantation immunity. *Nature* **171**: 267.
2. BILLINGHAM, R. E., L. BRENT & P. B. MEDAWAR. 1954. Quantitative studies on tissue transplantation immunity. II. The origin, strength and duration of actively and adoptively acquired immunity. *Proc. Roy. Soc. London, B.* **143**: 58.
3. MEDAWAR, P. B. 1946. Immunity to homologous grafted skin. II. The relationship between the antigens of blood and skin. *Brit. J. Exptl. Pathol.* **27**: 15.
4. DERICK, C. L. & H. F. SWIFT. 1929. Reactions of rabbits to nonhemolytic streptococci. I. General tuberculin-like hypersensitiveness, allergy, or hyperergy following the secondary reaction. *J. Exptl. Med.* **49**: 615.
5. JULIANELLE, L. A. 1930. Reactions of rabbits to intracutaneous injections of pneumococci and their products. *J. Exptl. Med.* **51**: 441, 449, 463, 625, 633 & 643.
6. DIENES, L. 1936. The specific immunity response and the healing of infectious diseases. Significance of active immunity and the connections between the immunity response and the anatomic lesions. *Arch. Pathol.* **21**: 357.
7. HECHT, R., M. B. SULZBERGER & H. WEIL. 1943. Studies in sensitization to skin. I. The production of antibodies to skin by means of the synergistic action of homologous skin antigen and staphylococcus toxin. *J. Exptl. Med.* **78**: 59.
8. INNES, J. R. M. 1950. Experimental allergic encephalomyelitis and implications regarding the aetiology of demyelinating diseases of man and animals. *Brit. Vet. J.* **106**: 93.
9. HURST, E. W. 1952. Experimental demyelination in relation to human and animal disease. *Am. J. Med.* **12**: 547.
10. WAKSMAN, B. H. & R. D. ADAMS. 1955. Allergic neuritis: an experimental disease of rabbits induced by the injection of peripheral nervous tissue and adjuvants. *J. Exptl. Med.* **102**: 213.
11. MÜLLER, H. 1952. Tierexperimentelle Untersuchungen zur Ophthalmia phakogenetica. Albrecht von Graefe's Arch. Ophthalmol. **153**: 1.
12. COLLINS, R. C. 1949. Experimental studies on sympathetic ophthalmia. *Am. J. Ophthalmol.* **32**: 1687.
13. COLLINS, R. C. 1953. Further experimental studies on sympathetic ophthalmia. *Am. J. Ophthalmol.* **36** (Part II): 150.
14. VOISIN, G., A. DELAUNAY & M. BARBER. 1951. Sur des lésions testiculaires provoquées chez le cobaye par iso- et auto-sensibilisation. *Ann. inst. Pasteur.* **81**: 48.
15. FREUND, J., M. M. LIPTON & G. E. THOMPSON. 1953. Aspermatogenesis in the guinea pig induced by testicular tissue and adjuvants. *J. Exptl. Med.* **97**: 711.
16. ROSE, N. R. & E. WITEBSKY. 1956. Studies on organ specificity. V. Changes in the thyroid glands of rabbits following active immunization with rabbit thyroid extracts. *J. Immunol.* **76**: 417.
17. JOY, H. H. 1953. Sympathetic ophthalmia. The history of its pathogenic studies. *Am. J. Ophthalmol.* **36**: 1100.
18. COWPER, A. R. 1951. Harada's disease and Vogt-Koyanagi syndrome—uveoencephalitis. *Arch. Ophthalmol. Chicago.* **45**: 367.
19. SUIE, T. 1955. An immunologic study of rabbits sensitized with homologous uveal tissue. *Am. J. Ophthalmol.* **39**: 377.
20. BULLINGTON, S. J. & B. H. WAKSMAN. 1956. In preparation.

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